# Development of New Reporter Systems for Studying Gamma Butyrolactone Mediated Signaling in *Streptomyces*

**A THESIS**
**SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL**
**OF THE UNIVERSITY OF MINNESOTA**
**BY**

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**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS**
**FOR THE DEGREE OF**
**Master of Science**

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**June, 2014**

# Acknowledgements

I would like to acknowledge my adviser, Dr. Christine Salomon for her guidance and support during the course of this thesis. I would also like to thank Dr. Eriko Takano and Dr. Linda Kinkel for their contributions towards this project. I am obliged to Dr. Gary Dunny for providing directions which helped me immensely in improving this thesis. I am grateful to Dr. Dan Schlatter and Chris Gelbmann for training me and Dr. Jon Henrikson for his assistance in this work.

# Dedication

To my mom, Govind Saraswat and all my dear friends who motivated me in this journey.

## Abstract

***Streptomyces*** are saprophytic bacteria ubiquitous in soil and well-known as producers of two-thirds of clinically used antibiotics. Antibiotic production and in some cases morphological differentiation is regulated by population density dependent signaling. Among the signaling molecules that have been identified, $\gamma$-butyrolactones (GBLs) form the major class. GBLs are small diffusible molecules which function at nanomolar concentrations and three major structural types are known. Due to limited quantities of GBLs produced, the traditional approach of isolation and structure determination followed by the study of functional roles is challenging. The development of two new receptor based reporter systems for A-factor and Virginiae Butanolide type GBLs is reported here. Together with a previously developed reporter for SCB type GBLs, these reporters can be utilized for screening environmental samples and pure compounds for signaling activity. They are useful for studying the specificity of various GBL signal-receptor systems among ***Streptomyces*** and to identify potential new signaling molecules. The reporters also serve as valuable tools in the study of GBL signal based communication between ***Streptomyces*** and other species and contribute towards a broader understanding of the role of signaling in ***Streptomyces*** ecology and evolution. Even though the development of fully functional reporters could not be completed, this study provides valuable insight into the many issues associated with developing ***Streptomyces*** based reporter systems. Modifications to the reporter system that may address these issues are discussed. Further, results from preliminary studies conducted for developing high throughput methods for screening signaling activity among ***Streptomyces*** isolates are also reported.

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# Chapter 1

# Background and Significance

Bacteria use chemical signaling molecules to mediate their interactions with the environment as well as with members of their own or different species. A chemical signaling molecule is defined as a metabolite from one cell which induces a change in other cells that is not due to the metabolism of the signaling molecule [56]. It has been shown that bacterial characteristics such as virulence, antibiotic production, biofilm formation, symbiosis, competence, conjugation and motility are community behaviors facilitated by a type of signaling known as population density-dependent signaling or Quorum Sensing [8]. Population density-dependent signaling is presumed to have evolved due to the effectiveness of a community based response to an external disturbance as opposed to the response of a single cell [62]. The concentration of signaling molecules present gives an indirect measure of the cell density of the producer species and as a result, at high cell densities certain beneficial behaviors can be cooperatively turned on more effectively by the signal receivers (see Figure 1.1 for a schematic representation). The study of microbial signaling is an emerging field and the structures of the signaling molecules identified in the known interactions are found to be varied, Acyl Homoserine Lactone (AHL) type molecules are commonly found among Gram-negative bacteria, modified oligopeptides among Gram-positive bacteria and autoinducer-2 (AI-2) signaling system found in many Gram-positive and Gram-negative bacteria. [62].

Research on signaling systems indicates that signaling occurs mostly between closely related species and that signal-receiver interactions are highly specific, presumably to prevent non-signal producers from taking undue advantage of the outcome of signaling

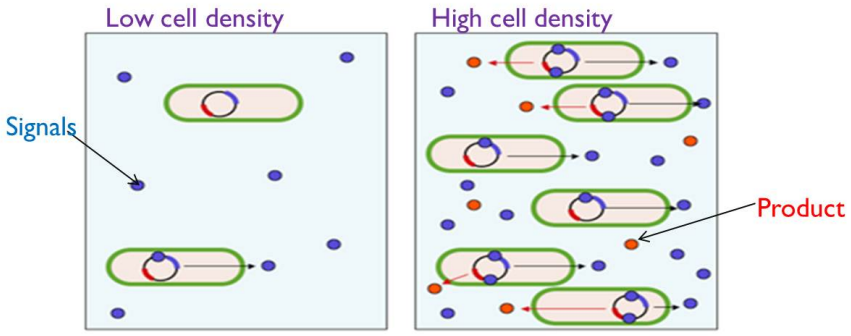



Figure 1.1: Population density dependent signaling. At low cell density, signaling molecules secreted by cells are present in the environment at low concentrations. A critical concentration of the signaling molecule is reached at high cell densities and this causes certain genes to be "turned on", resulting in the concerted production of the gene product.

[50]. Despite this, myriad types of interactions have also been observed with examples found in literature for eavesdropping, in which a signal produced by one species is used as a cue by another and cross-talk between two species due to the overlap between signals of the producing and receiving species [12] [38] [30]. Recent research has also suggested that signaling molecules mediate inter-kingdom interactions between soil bacteria and plants [24] [35].

Ecological implications of these signaling interactions and how species interactions have driven the evolution of signaling is a growing research topic. Various aspects of signaling in terms of the frequency of signaling, the specificity of signaling molecules and receptors, the identity of various types of signaling molecules and the functions of these interactions in the environment need to be further investigated. Another important reason to pursue the study of signaling molecules is the potential it offers to understand the regulation of important microbial behaviors such as antibiotic production and virulence. Developing robust and sensitive tools for signal molecule detection is integral to the discovery of new signaling molecules and to identify additional roles of signaling molecules. These detection systems when used in large-scale screening studies can provide valuable information about the signal producers and receivers that will shed light on the dynamics of signal-mediated communication in various ecosystems. For example, the reporters may be used to screen a wide variety of samples of any origin - microbial, plant or animal and detect the presence of specific signaling compounds. They may also

be utilized to test isolates from various geographically separated microbial communities to learn patterns of signaling among various proximate species, as well as factors that influence signaling and its effect on microbial behaviors.

This chapter introduces $\gamma$-butyrolactone (GBL) signaling systems found among *Streptomyces*. The mechanism and role of GBL signaling, the structure of signaling molecules and the methods used for detecting GBLs are discussed. The challenges associated with the discovery of GBLs, the motivation for developing reporter systems and applications of the reporters are also explained. A reporter system developed by Hsiao *et al.* for detecting SCB-type GBLs found in *Streptomyces coelicolor* and their findings using this reporter is discussed in detail. The development of similar reporter systems for A-factor and Virginiae butanolide (VB) signaling compounds found in *Streptomyces griseus* and *Streptomyces virginiae*, respectively, is presented in this thesis. A brief literature review of A-factor and VB signaling systems is also presented. In Chapter 2, methods used for developing the new reporters and conducting the bioassays are described. Chapter 3 examines the results of this study and Chapter 4 presents the conclusions and future directions.

## 1.1 Signaling in *Streptomyces*

*Streptomyces* is a genus of approximately 550 species of aerobic, gram positive, non-motile, high G+C bacteria. They are found ubiquitously in bulk soil and marine sediments, and are associated with plants as endophytes and members of rhizosphere communities. *Streptomyces* undergo a complex developmental life cycle and the morphological differentiation stages are often closely associated with the production of secondary metabolites [19]. Close symbiotic associations of *Streptomyces* with plants, fungi and bacteria have been observed and may have evolved as a direct result of their ability to produce these secondary metabolites [53]. Antibiotic synthesis, turned on in the stationary growth phase, is generally believed to be a defense mechanism to prevent motile, competing species from exploiting their food resources [9].

About two-thirds of all clinically used natural antibiotics are produced by *Streptomyces* [63]. Some examples of clinically important *Streptomyces* antibiotics are streptomycin, vancomycin, chloramphenicol and avermectin which are used for targeting

diseases ranging from typhoid and tuberculosis to cancer [9]. The antibiotic synthesis genes in *Streptomyces* are mostly arranged in clusters under the control of pathway specific regulatory genes [7]. Genomic studies of several *Streptomyces* spp. have shown the presence of many such clusters of as yet unknown activity [57]. This suggests their potential to produce many more antibiotics, making them an economically important genus. GBLs ($\gamma$-butyrolactones) coordinate the production of antibiotics and in some cases sporulation in *Streptomyces*. They act as pleiotropic regulators or higher level regulators of the pathway specific regulatory genes and are consequently studied for their involvement in the regulation of antibiotic and other secondary metabolite synthesis.

GBLs are small, hydrophobic molecules synthesized in nanomolar quantities which can easily diffuse through membranes into the extracellular environment. The first GBL discovered from *Streptomyces* was A-factor, which induces streptomycin production and sporulation in *Streptomyces griseus* [32]. Subsequently, additional GBL structures were reported from other actinomycetes that also play a role in antibiotic production and morphological differentiation [10]. To date, structures of 14 different GBLs from seven species of *Streptomyces* have been identified (Figure 1.2). GBL compounds are divided into three major structural types depending on the bond at the C-6 position: 6-keto type ( e.g. A-factor), (6R)-hydroxy type ( e.g. SCB1-3, IM-2 ) and (6S)- hydroxy type (e.g. virginiae butanolides A - E) [19]. Bacteria that recognize GBL signals are exquisitely sensitive to their cognate structures. For example, nanomolar concentrations of A-factor and SCB1 induce the production of actinorhodin in *S.griseus* and undecylpordigiosin in *S.coelicolor* respectively [16] [58]. The extremely small quantities in which GBLs are produced, their sensitivity and closely related structures indicate the highly specific nature of this signaling process.

A number of other signaling molecules which regulate both morphogenesis and antibiotic production have also been isolated from *Streptomyces*. Methylenomycin Furans (MMFs), siderophores and peptides like SapB are a few examples [64]. More recently, butenolides such as avenolide from *S.avermitilis* and SRB1 and SRB2 from *S.rochei* were identified as inducers of antibiotic synthesis in these species [3] [34]. These butenolides are structurally slightly different from GBLs, but specifically induce antibiotic production in the producing species. GBLs still remain the largest group of signaling molecules discovered in *Streptomyces* and are generally assumed to be the more common form of

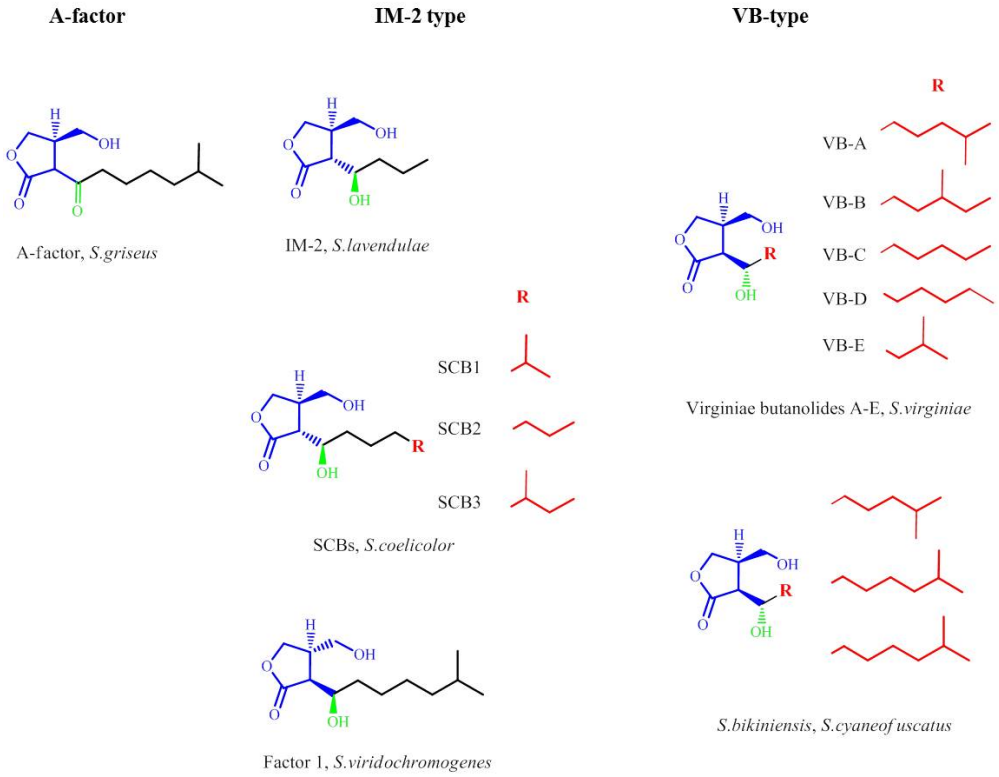



Figure 1.2: The structure, type, and producing species of the 14 GBLs identified so far. The functional group at the C-6 position (shown in green) differentiates the structures into 3 distinct types: A-factor type, IM-2 type and VB type. The common structural elements are given in blue and the length and branching variations in side chains are shown in red.

signaling in *Streptomyces* [65]. Extensive research has been done on understanding how GBL signaling operates and many of the pathways and genes involved have been identified. The mechanism of action of non-GBL signaling molecules and their regulation are only beginning to be understood.

## 1.2 GBL Signaling Mechanism

In *Streptomyces*, genes involved in antibiotic synthesis occur as clusters which are held under transcriptional control of pathway specific regulatory genes [7]. GBL signaling molecules induce antibiotic production by binding specifically to the receptor protein

which is the transcriptional repressor for antibiotic synthesis. The binding causes conformational changes in the repressor protein so that it can no longer bind to the promoter DNA of the antibiotic cluster [57]. The relief of repression by the signaling molecule thus results in antibiotic synthesis. This mechanism is illustrated in Figure 1.3. Most GBLs negatively regulate their own synthesis as well so that at any point in time the concentration of GBLs in an environment is a reflection of the population density of the producing species [57]. Only when a critical concentration is reached does the signaling cascade become activated.

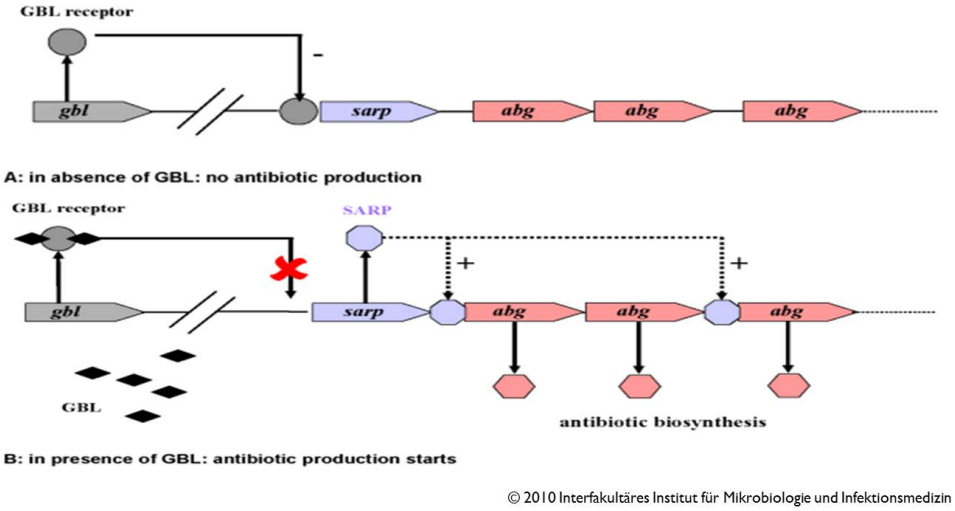



Figure 1.3: Mechanism of action of GBLs. Here the *Streptomyces* antibiotic biosynthetic genes (abg) are depicted as a cluster which is under the regulation of a single *Streptomyces* Antibiotic Regulatory Protein (SARP). In the absence of GBL, expression of SARP is repressed by the GBL receptor protein. In the presence of GBL, repression is relieved by the binding of GBL to the GBL receptor and the SARP activates the abg genes which culminate in the production of antibiotic. Image from Mast *et al.* [40]

## 1.3 Search for GBL-like Molecules That Can Bind to GBL Receptors

Homology searches of the known GBL receptor and synthase genes show that a number of *Streptomyces* species carry homologs of these genes, indicating the presence of putative GBL signaling systems [57]. However, the extremely small quantities in which GBLs are produced make their isolation and detection challenging [57]. Hundreds of liters of

bacterial culture must be grown and purified through High Performance Liquid Chromatography (HPLC) to obtain microgram quantities of the GBL. The limited quantity of molecules available makes the downstream structure elucidation by techniques like Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) challenging. Hence, a number of alternative approaches have been designed for the study of GBLs.

The most widely used assay for the detection of GBLs is via the induction of antibiotic production since this is the most common end result of the signal-receptor binding cascade. Antibiotic production can be monitored visually (in the case of colored antibiotics), spectrophotometrically or through bacterial growth inhibition assays [58]. Even though this method is simple and convenient, there are several disadvantages. Although the signal-receptor binding triggers antibiotic synthesis, a large number of other factors are involved in the ultimate expression of antibiotic thereby affecting the validity of the method. Also, parameters such as inoculum concentration, duration of cultivation, media, growing conditions etc. affect the reproducibility of these assays [23]. Therefore, methods which can directly detect signal-receptor binding are considered to be more sensitive and reliable [23]. In addition to detecting GBLs, direct GBL receptor protein-based methods can be utilized for studying the binding of cognate and non-cognate molecules to GBL receptors, thereby establishing the sensitivity and specificity of the receptor proteins and discover the interaction patterns among *Streptomyces* species.

In this category, several new approaches both *in vivo* and *in vitro* have been established such as His-tagged GBL receptors coupled with mass spectrometry (MS), cell-free translation with GFP expression coupled to the GBL receptor binding gene, the GBL receptor protein affinity capture matrix coupled with tandem mass spectrometry and kanamycin resistance expression coupled to the GBL receptor binding gene [69] [23] [70] [25]. Although these methods measure direct binding of GBLs to the receptors, there are some significant drawbacks. The mass spectrometric methods give direct structural information but are challenging to perform and can only be used to detect the most abundant analog of GBL present in the sample, which in most cases also has the lowest binding affinity to the corresponding receptor [69]. The cell-free translation method developed in *E.coli* was used to measure the binding of non-cognate signals C6-Homoserine lactone and VB-C to the SCB receptor protein [70]. The SCB receptor protein based

kanamycin resistance bioassay developed by Hsiao *et al.* is also a similar, simpler system and successfully showed the binding of 18 non-cognate synthetic GBLs and signals from extracts of two *Streptomyces* isolates to the SCB receptor protein [23]. Because of the promising results obtained using this system, which is described in detail in the following section, this project was undertaken to develop similar detection systems for the other two structural classes of GBL molecules: A-factor type and VB type.

## 1.4 Kanamycin Resistance Bioassay Using the SCB Receptor Protein Based Reporter

Hsiao *et al.* modeled a reporter system based on the actual GBL signaling cascade. A promoterless kanamycin resistance gene is fused to the promoter of the gene repressed by the GBL receptor so that the expression of the kanamycin resistance gene is repressed in the absence of the GBL. In the presence of the GBL, the binding of the GBL to the receptor relieves the repression and causes kanamycin resistance gene to be expressed. Thus, a reporter strain which carries these genes will be able to grow in the presence of kanamycin only when a signaling molecule that can bind to the receptor is present.

A host strain which cannot produce or detect signals is also required to prevent the interference from natural signaling molecules and receptors. Hsiao *et al.* developed a host strain, LW16, by disrupting *scbA* gene which is the key gene involved in *Streptomyces coelicolor* Butyrolactone (SCB) biosynthesis and *scbR* gene which encodes the SCB receptor protein in *S.coelicolor* [23]. Since *Streptomyces* do not stably maintain recombinant plasmids, an integrating vector pIJ82, was designed by Hsaio *et al.* for the chromosomal integration. The plasmid pIJ82 is a *Streptomyces* phage-derived vector containing the *int* gene which encodes an integrase enzyme enabling it to integrate into *S.coelicolor* genome based on specific attachment sites. The recombinant vector pTE134 (see Figure 1.4) was then constructed from the plasmid pIJ82 by inserting *scbR* gene and a promoterless kanamycin resistance gene, *neo*, fused to the *cpkO* promoter. The *cpkO* promoter is one of the native DNA targets of ScbR in *S.coelicolor* and its binding regulates the expression of a Type I polyketide antibiotic cluster of unknown function. The plasmid pTE134 was introduced into host strain LW16 to create the SCB reporter (LW16::pTE134). The SCB reporter can be used in the kanamycin resistance bioassay

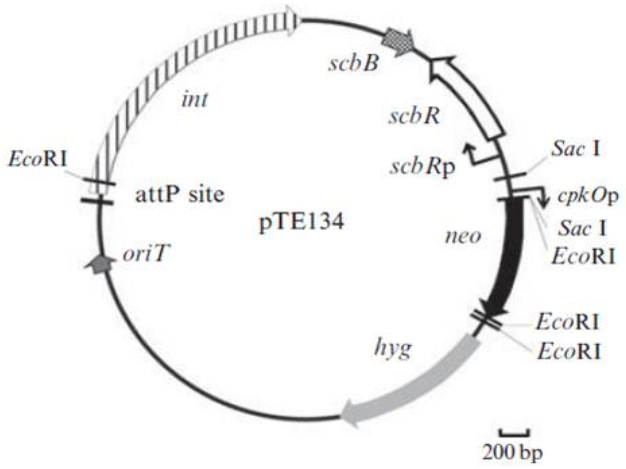



Figure 1.4: Map of pTE134, the plasmid developed for the SCB reporter kanamycin resistance bioassay. The plasmid pTE134 carries the *scbR* with its own promoter region, *scbRp* and a *cpkO* promoter, *cpkOp*, coupled with a promoterless kanamycin resistant gene, *neo*. It also has the hygromycin resistance gene, *hyg*, as the selectable marker for the plasmid and the *int* gene (phiC31 integrase) for chromosomal integration of plasmid based on *attP* attachment site (phiC31 attP site). The plasmid pTE134 was introduced into *S.coelicolor* LW16 to create the SCB reporter. (Image taken from Hsiao *et al.* [23])

to detect SCBs and structurally related GBLs, presumably of the SCB type. In the absence of a suitable signaling molecule, ScbR will repress the *neo* gene and the reporter will be susceptible to kanamycin. The ability of the reporter to grow in the presence of kanamycin will indicate the presence of signaling molecules capable of binding to ScbR (Figure 1.5).

## 1.5 The Scope of the Kanamycin Resistance Bioassay Using the SCB Reporter

The advantages of the kanamycin resistance bioassay are that it is simple, efficient and provides fast results. It gives clear positive results with the size of the halo corresponding (although not linearly) to the amount of signaling molecule spotted. Also, the principle on which it works is direct and follows the natural signaling mechanism of GBLs. Signals which weakly bind to the receptor protein and may not give detectable antibiotic production in antibiotic assays can give positive results in this assay. Hsiao

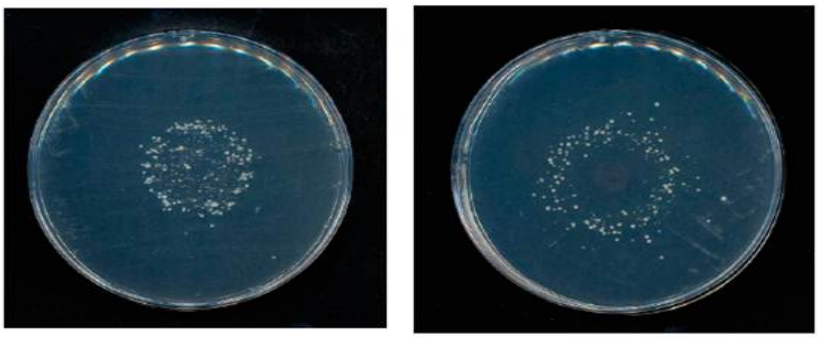


Figure 1.5: Kanamycin resistance bioassay results when tested with pure synthetic SCB1 (left) and the extract from wild type *S.coelicolor*, M145 (right). The SCB reporter spores were plated on the agar medium containing kanamycin and the compound/extract spotted in the center. Following incubation, the SCB reporter spores were observed to grow around the area in which the signaling molecules diffused. (Image from Hsiao *et al.* [23])

*et al.* successfully used the SCB reporter as a screening tool for assessing the binding of native and non-native synthetic GBLs to ScbR. They found that SCB3, one of the three SCBs isolated from *S.coelicolor*, was the most effective in binding to ScbR. Only 0.006 $\mu$g of SCB3 was required to obtain a positive result in the kanamycin resistance bioassay. In contrast, A-factor which is not known to be produced by *S.coelicolor*, only induced a positive response at 0.625 $\mu$g indicating a significantly weaker binding affinity. The results of Hsiao *et al.* from screening other synthetic GBLs with the SCB reporter are shown in Table 1.1. Hsiao *et al.* also tested the reporter with synthetic analogs of the three types of GBLs, varying in their chain lengths and branching. They found that the branching at the C-6 position is the preferred ligand to ScbR [23]. The kanamycin resistance bioassay with the SCB reporter demonstrated an improved ability to detect non-cognate signals that bind to ScbR compared to the indirect antibiotic induction assays. The lower detection limit makes it a promising tool for studying the binding of non-cognate ligands to GBL receptors [23].

The SCB reporter also provides a good model system for developing new reporters for other GBL receptors. A-factor type and VB type GBLs form the remaining two known GBL structural types and the genes involved in their regulation are known. This makes the development of reporters for these systems based on the SCB reporter

| Synthetic GBL | Minimum Concentration (µg) |
|---|---|
| SCB1 | 0.025 |
| SCB2 | 0.05 |
| SCB3 | 0.025 |
| A-factor | 1.25 |
| VB-C | - |
| VB-D | 6.25 |
| IM-2 | - |

Table 1.1: Minimum concentrations of synthetic racemic GBLs that activated the SCB reporter (Data from Hsiao *et al.*[23]). The minus sign indicates that no halo was observed on the plate at the concentrations tested. The highest concentration tested was 62.5 $\mu$g for VB-C and 31.25 $\mu$g for IM-2.

model comparatively simple. Since the *S.coelicolor* host strain, LW16, used in the SCB reporter does not produce A-factor or VB signals, it can be utilized as the host strain for new reporters as well. Employing similar detection systems also ensures the comparability of results obtained with different reporters. The SCB reporter was successful in showing that non-cognate signals can bind to the SCB receptor protein. Developing new reporters based on the other GBLs will help elucidate more aspects of GBL signaling, including how effectively a single type of signaling molecule may bind to multiple receptors and vice versa. The SCB reporter together with new reporters for A-factor and VB systems will be ideal tools for examining the communication patterns among *Streptomyces* sp. through signals encompassing all three GBL types and provide more comprehensive understanding of its role in interspecies, intraspecies and inter-kingdom interactions.

## 1.6 A-factor System

A-factor, also called autoregulatory factor (2-isocapryloyl-3R-hydroxymethyl-$\gamma$- butyrolactone), from *Streptomyces griseus* was the first GBL signaling molecule to be discovered from *Streptomyces*. Most GBLs regulate only secondary metabolite production but A-factor induces sporulation, streptomycin biosynthesis and production of a yellow pigment, grixazone, in *S.griseus* and hence it can be considered as a representative of GBL-type regulators which control both morphological and physiological differentiation

[32] [18] [47].

In liquid culture, A-factor is produced in a growth dependent manner with concentrations reaching 25 ng/ml at the end of exponential phase [16]. After this critical concentration is reached, the amount of A-factor rapidly decreases due to a cease in its production coupled with the instability of A-factor in water and other media [2] [18]. Horinouchi *et al.* has given an excellent illustration of the time scale of A-factor production in relation to growth and secondary metabolite production in *S.griseus* (see Figure 1.6) [18]. Timing is important for the inducing function of A-factor since commitment to sporulation and streptomycin biosynthesis are determined early in the development cycle, usually 10-12 hours after inoculation [5] [45]. This period is called the decision phase or A-factor sensitive period since after this point addition of A-factor to the media will not affect morphological or physiological differentiation [2] [45] .

The complete biosynthetic pathway of A-factor has been elucidated and the gene *afsA* which encodes the key enzyme in the the biosynthetic pathway has been identified and characterized [26] [20] [21]. AfsA, a 301 amino acid protein, catalyzes the acyl transfer between a C10 $\beta$-ketoacyl bound acyl carrier protein (ACP), which is one of the intermediates in fatty acid biosynthesis, and the hydroxy group of dihydroxyacetone phosphate (DHAP) [26]. The fatty acid ester product of this reaction can then undergo simple condensation and reduction reactions to form A-factor [26]. Apart from AfsA, the rest of the components in the A-factor biosynthetic pathway are common to most bacterial cells and it has been shown that the introduction of *afsA* results in the synthesis of A-factor analogs in non- A-factor producing *Streptomyces* species and even in *E.coli* [1]. A-factor production is limited by the small amount of the AfsA substrate, C10 $\beta$-ketoacyl-ACP, leaked from the fatty acid biosynthetic pathway as well as the presence of a regulatory feedback loop for *afsA* expression [19]. The *afsA* gene is located towards the end of a linear chromosome, approximately 100 kb away from the streptomycin biosynthetic gene cluster and due to the propensity of the ends of *Streptomyces* chromosomes to undergo large-scale mutations, A-factor production is found to be unstable [39].

A-factor activates a signaling cascade by binding to its specific receptor named A-factor Receptor Protein (ArpA), which is a repressor protein that regulates expression of A-factor Dependent Protein (AdpA). AdpA in turn targets a host of genes involved

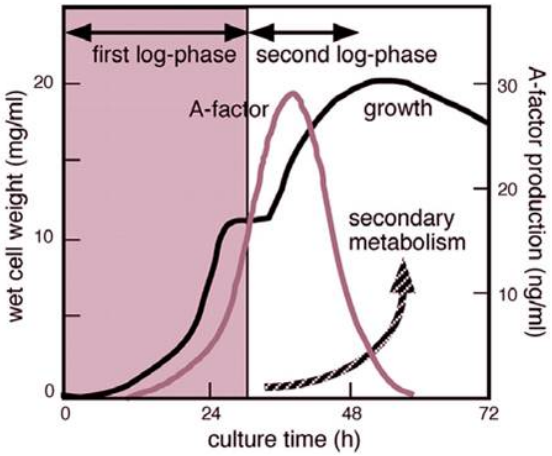



Figure 1.6: Production of A-factor in *S.griseus* in relation to growth and secondary metabolite (streptomycin) production. A-factor is produced in a growth-dependent manner and accumulates until it reaches a critical concentration. Streptomycin production is initiated at this point and is followed by the disappearance of A-factor. (Image from Horinouchi *et al.* [18])

in the morphological and physiological differentiation, termed as the AdpA regulon (see Figure 1.7). In the absence of A-factor, ArpA binds to the promoter region of AdpA and acts as the transcriptional repressor.

ArpA is a 276 amino acid protein encoded by the gene *arpA* and it binds to A-factor with 1:1 molar ratio and a very low dissociation constant, Kd, of 0.7 nM [41]. The crystal structure of ArpA has not been elucidated because of its tendency to aggregate in solution, but CprB, a homolog of ArpA in *S.coelicolor*, has been crystallized and structural comparisons suggest that they belong to TetR family of proteins [19] [44].

AdpA is a 405 amino acid protein which binds to the promoter regions of its target genes and initiates transcription by recruiting the RNA polymerase and facilitating isomerization of the RNA polymerase-DNA complex [48] [68]. Though the effect of AdpA on the streptomycin biosynthesis, aerial mycelium formation and regulation of its own transcription has been studied, knowledge of its regulation of other genes is limited [60] [67] [27]. Studies indicate that AdpA is a global transcriptional activator influencing the expression of more than 1000 genes in *S.griseus* [17]. In recent years, genome analysis as well as crystallographic studies of AdpA DNA binding domain has been performed towards the purpose of understanding the genes involved in AdpA regulon and its regulatory mechanism [71]

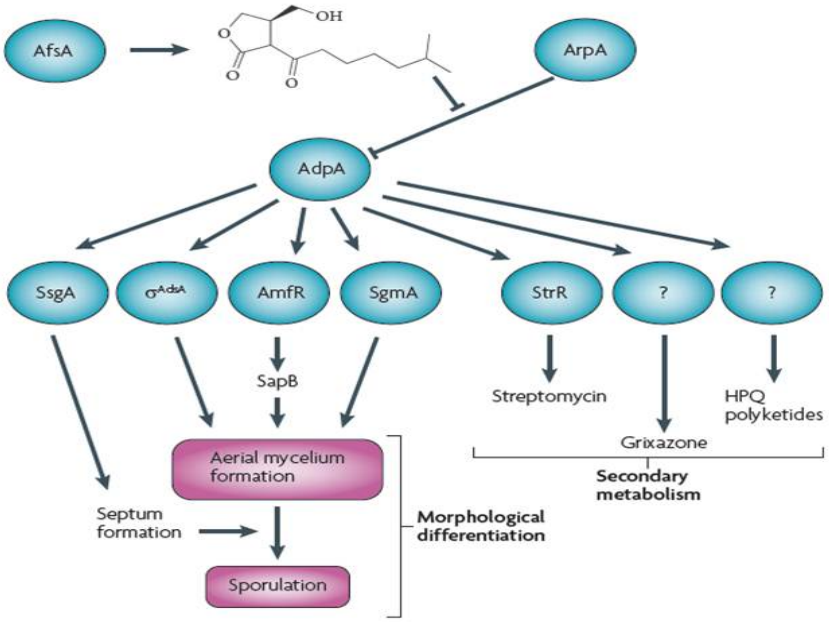



Figure 1.7: A-factor regulatory cascade in *S.griseus*. AfsA is the key enzyme in the A-factor biosynthesis. In the presence of A-factor, the receptor protein, ArpA, is unable to repress AdpA expression. AdpA directly regulates the expression of a host of genes involved in the morphological and physiological differentiation. (Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] (Flardh *et al.* [13]), copyright (2009).

## 1.7 Virginiae Butanolide System

Isolation and structural determination of virginiae butanolides (VBs) from *Streptomyces virginiae* closely followed the discovery of A-factor [66] [36]. The VBs A-E differ in the length and branching of their side chain (Figure 1.2). All induce the coordinated production of two different antibiotics, virginiamycins M and S in *S.virginiae* at nanomolar concentration [66].

Until recently, the role of the AfsA homolog in *S.virginiae*, BarX, in VB biosynthesis was unclear with some results suggesting it exhibited only a regulatory role [28]. However, support for its enzymatic activity has emerged since and it has been shown that AfsA is capable of functionally complementing BarX in a *barX*-null mutant [37]. Though the VB biosynthetic pathway is not as clearly understood as the A-factor biosynthesis, feeding experiments and genetic studies indicate that VB biosynthesis involves a different pathway from that of A-factor. The key difference is that instead

of butenolide phosphate, DHAP (the substrate of AfsA), the substrate for BarX is a butenolide, dihydroxy acetone (DHA). This results in the formation of a $\beta$-keto ester as opposed to $\beta$-keto acid formed in the A-factor biosynthesis [51]. Also, unlike A-factor, the keto group at C-6 is reduced to a hydroxyl group in VBs and the enzyme required for catalyzing this additional reaction was identified as BarS1 [55].

Another major difference between A-factor and VB systems is that unlike *afsA*, *barX* is located just upstream of its butyrolactone autoregulator receptor, BarA. BarA is a 232 amino acid protein almost exclusively present in the cytoplasm and is encoded by the *barA* gene [65]. The regulatory cascade resulting from the binding of VB to BarA which results in the induction of virginiamycin production in *S.virginiae* is not yet completely understood, but the genes involved are found to be clustered in a 10 kb region known as the virginiamycin regulatory island in the 77 kb virginiamycin biosynthetic gene cluster [49] .

Binding of VB to BarA relieves the repression of *barB*, *varS* and *varM* genes [43]. BarA acts as a repressor for *barX* as well, thus repressing VB biosynthesis [42]. The transcription of *varS* and *varM* allows cells to acquire virginiamycin resistance while BarB temporarily represses the virginiamycin pathway specific regulatory gene, *vmsR*, thereby delaying the virginiamycin biosynthesis [28]. BarA appears to transciptionally control the *vmsR* expression as well [29]. VmsR, a SARP (*Streptomyces* Antibiotic Regulatory Protein) like protein, is in turn a regulator for regulatory genes which directly control virginiamycin biosynthetic genes. A complete picture of the VB regulatory cascade has not yet emerged, but it appears that BarA and BarX are higher level regulators and both involved in a number of cell functions, including virginiamycin biosynthesis and virginiamycin resistance [49].

## 1.8 Reporters for A-factor and VB

Antibiotic production is the assay currently used for studying the signaling activity of A-factor and VB type signaling molecules. A-factor and its various synthetic analogs were tested for biological activity in *S.griseus* by the induction of streptomycin production [31]. It was observed that acyl derivatives of A-factor exhibited high biological activity comparable to A-factor. GBLs from strains *S.coelicolor* and *S.viridochromogenes*, both

IM-2 type GBL producers, were also found to be active in this assay with activity 2-3 fold less than A-factor. Similarly, synthetic analogs of VB were tested for their capability to induce virginiamycin in *S.virginiae* [46]. Among 41 synthetic VB analogs tested, it was observed that VB-C type compounds with heptyl and octyl side chains were 2.5 fold more active than VB-C. A-factor type analogs were also tested for biological activity in this assay and were found to induce 10 fold lower virginiamycin production than VB-C.

These results point to the specificity as well as the flexibility of the A-factor receptor, ArpA, and the VB receptor, BarA, in detecting cognate and non-cognate signals. In all instances it was found that receptors were most sensitive towards their cognate GBLs, while some synthetic GBL derivatives and natural, non-cognate GBLs activate receptors at higher concentrations. It is interesting to note that IM-2 type GBLs (SCB, Factor 1) appear to be able to bind to the A-factor receptor and A-factor type GBLs are capable of binding to the VB receptor. However, studies on the specificity of the binding of different GBL types to the GBL receptors of each type is incomplete. For example, the binding of IM-2 type GBLs with the VB receptor and that of VB type GBLs with the A-factor receptor is not known. The different methodologies that were followed for the detection and quantification of the binding to the different GBL receptors also complicate comparison of results from different studies. Furthermore, the frequency of signaling among *Streptomyces* through different types of GBLs and their role in *Streptomyces* in natural environments has not yet been studied.

As discussed in previous section, antibiotic assays utilized in these GBL studies have several drawbacks that limits their applications. On the other hand, the SCB reporter assay for detecting IM-2 type GBL, SCB, was successfully used to study the binding of all natural GBL types and their synthetic derivatives to the SCB receptor protein. The simple format of this assay also allows for large-scale screenings to study the ecological role of signaling. Therefore, similar assays which can detect the signaling of A-factor and VB type GBL molecules by directly monitoring the binding to the receptor proteins need to be developed for conducting meaningful comparative studies of the three types of GBLs.

# Chapter 2

# Development of New A-factor and VB Reporters

The new reporter systems for A-factor and VB described here were developed utilizing the existing framework established by Hsiao *et al.* for the SCB reporter. Since the SCB reporter utilized the fundamental GBL signaling mechanism as the basis for the assay, this method was adapted to develop similar reporters for A-factor and VB type GBLs. The *scbR* gene encoding the SCB receptor protein in the SCB reporter was substituted with the orthologous genes *arpA* and *barA* which encodes the A-factor receptor protein, ArpA, and VB receptor protein, BarA, respectively. Likewise, analogous to the *cpkO* promoter region which acts as the target for ScbR in the SCB reporter, the promoter region of AdpA was used as the DNA target for ArpA and the promoter region of BarB as the target for BarA.

The cloning protocol, PCR amplified fragments of *S.griseus arpA*, promoter region of *adpA* and *S.virginiae barA*, primers used to amplify these fragments, synthetic oligonucleotides corresponding to promoter regions of *barB* and the plasmid vectors used in the cloning, pIJ82 and pTE133, were kindly provided by Dr. Eriko Takano's lab at University of Groningen, Netherlands.

## 2.1 Reagents

The pGEM-T Easy vector system and GoTaq Green Master Mix for Polymerase Chain Reactions (PCR) were purchased from Promega (Madison, WI). Restriction enzymes, T4 DNA ligase, T4 Polynucleotide Kinase, Phusion High-Fidelity DNA Polymerase and Antarctic phosphatase were obtained from New England Biolabs (Ipswich, MA). Kits for gel purification and plasmid mini preps were purchased from Qiagen (Valencia, CA) and Invitrogen (Grand Island, NY), respectively. The UltraClean Microbial DNA Isolation Kit from Mo Bio (Carlsbad, CA) was used for extracting genomic DNA from *Streptomyces*. Sanger DNA sequencing of all the samples was done at the Biomedical Genomics Center (BMGC, University of Minnesota). Primers were synthesized either at Sigma-Aldrich (St. Louis, MO) or the BMGC.

## 2.2 Bacterial Strains and Growth Conditions

All strains and plasmids used in this study are listed in Table 2.2. The *Streptomyces* strains were stored as 20% sterile glycerol spore stocks at $-20°$C and *E.coli* strains in 50% glycerol at -80°C. All *Streptomyces coelicolor* strains were grown at 30°C on the Soy Flour Mannitol (SFM [33]) agar medium for 5-7 days for spore stock preparation and on the SMMS agar (Supplemented Minimal Medium, Solid [33]) for the GBL extraction. *Streptomyces* strains were grown at 30°C with continuous shaking at 250 rpm for 3-5 days in the ISP2 broth for DNA isolation and in the Yeast Extract Malt Extract medium (YEME [33]) for measurement of optical density in liquid culture . The SCB reporter strain (LW16::pTE134) was grown under 50 $\mu$g/ml hygromycin selection and *S.coelicolor scbA scbR* double deletion mutant LW16 was grown on SFM with 50 $\mu$g/ml apramycin. *E.coli* ET12567/pUZ8002 was propagated in the LB medium with 50 $\mu$g/ml kanamycin and 25 $\mu$g/ml chloramphenicol. Competent cells of *E.coli* DH5$\alpha$ were prepared using the Inoue method for the preparation and transformation of *E.coli* ultra competent cells and plasmids pTE133 and pIJ82 were propagated by transforming into *E.coli* DH5$\alpha$ and selectively growing transformants on LB plates with 100 $\mu$g/ml ampicillin and 50 $\mu$g/ml hygromycin respectively [52].

## 2.3 Construction of A-factor and VB Reporter Strains

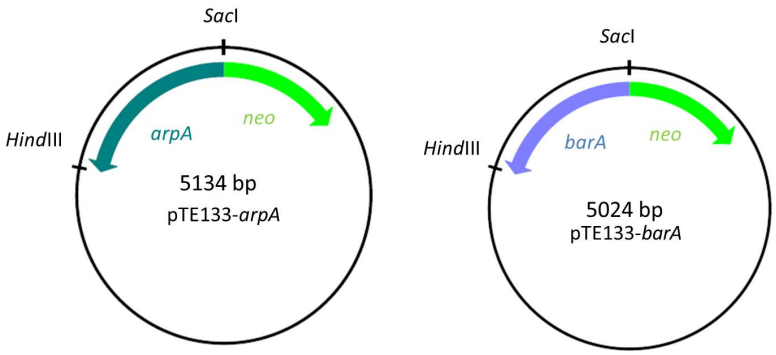
The *arpA*, promoter *adpA* and *barA* PCR products were first cloned into pGEM-T Easy vectors. The list of primers is given in Table 2.1. Since the PCR products were blunt ended, an A-tailing procedure as described in the pGEM-T cloning manual was performed prior to ligating with the pGEM-T Easy vector according to the cloning protocol. Several independent clones were selected and the correct insertion confirmed using PCR and subsequent sequencing of the purified PCR product in both forward and reverse directions using the appropriate primers.

### 2.3.1 Construction of pTE133-*arpA* and pTE133-*barA*

The plasmid pTE133 which carries a promoterless kanamycin gene, *neo*, was digested with the combination of enzymes HindIII and SacI. This reaction produced three fragments of lengths approximately 4 kb, 1257 bp and 75 bp. The digest mix was separated by electrophoresis in ultrapure agarose gel and the largest of the fragments (4 kb) was purified from the gel. The pGEM-T vectors containing *arpA* and *barA* genes were also digested with the combination of HindIII and SacI and *arpA* (1129 bp) and *barA* (1024 bp) fragments purified from the agarose gel. The purified DNA fragments were used in the ligation reaction using T4 DNA ligase and the ligation mix was used to transform *E.coli* DH5$\alpha$. The transformed clones were selected on LB-ampicillin plates and the successful insertion was confirmed by the DNA isolation and PCR amplification of inserted genes.

### 2.3.2 Introduction of promoter-*adpA* into pTE133-*arpA* and promoter-*barB* into pTE133-*barA*

The pTE133-*arpA* and pTE133-*barA* plasmids were digested with SacI to linearize the plasmid. To avoid the self-circularization of the plasmid during ligation step, the linearized vector was treated with Antarctic phosphatase. The pGEM-T vector carrying the promoter of AdpA gene was cleaved with SacI as well, giving rise to a 384 bp fragment which was purified through gel electrophoresis. Synthetic oligonucleotides for the promoter of BarB gene were designed so that sticky ends compatible with SacI overhangs remained after their annealing. To anneal the oligonucleotides, equimolar amounts of




(a) pTE133-*arpA* (b) pTE133-*barA*

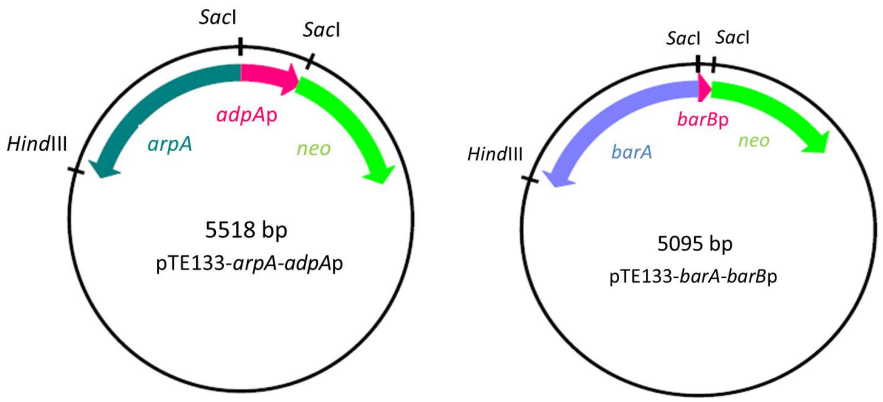
Figure 2.1: Schematic representation of pTE133-*arpA* and pTE133-*barA* plasmids showing the orientation of the A-factor receptor protein (ArpA) encoding gene, *arpA*, the VB receptor protein (BarA) encoding gene, *barA* and the kanamycin resistance gene, *neo*. HindIII and SacI restriction sites used in the cloning of genes are also shown.

forward and reverse molecules were mixed in a microcentrifuge tube, heated for 2-3 min at 95°C in the presence of T4 DNA ligase buffer and allowed to slowly cool down to the room temperature. The resulting double-strand molecules were treated with T4 Polynucleotide Kinase for addition of 5′-phosphates to allow subsequent ligation. The linearized pTE133-*arpA* and pTE133-*barA* were then ligated with promoter-*adpA* and the annealed promoter-*barB* respectively, and transformed into *E.coli* DH5$\alpha$.

This ligation resulted in the formation of two kinds of plasmids with the two genes ligated in either the same or opposing orientations. Since the repressor and promoter genes exist divergently in the native *Streptomyces* sp., the constructs with opposing orientation of the inserted genes were selected through PCR amplification with appropriate primer pairs.

### 2.3.3 Construction of integrating vector and introduction into LW16

The two plasmids obtained in the previous step were cleaved with BglII to release the cassettes containing the *arpA*/*barA* genes and the kanamycin resistance gene under control of the corresponding promoter and the cassettes were purified from the agarose gel. The integrating vector pIJ82 was cleaved with BamHI and treated with alkaline phosphatase. The linearized pIJ82 was then ligated with the cassettes and the recombinant

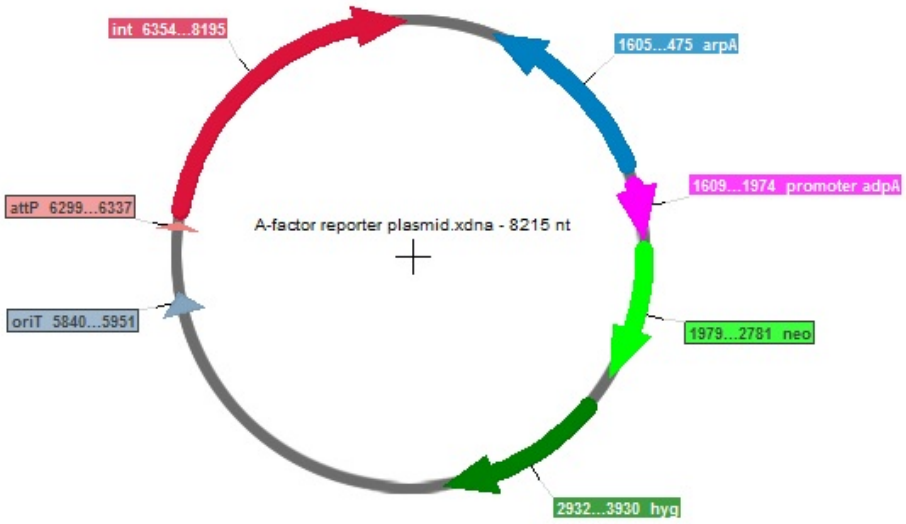



(a) pTE133-a*rpA*-promoter *adpA* (b) pTE133-*barA*-promoter *barB*

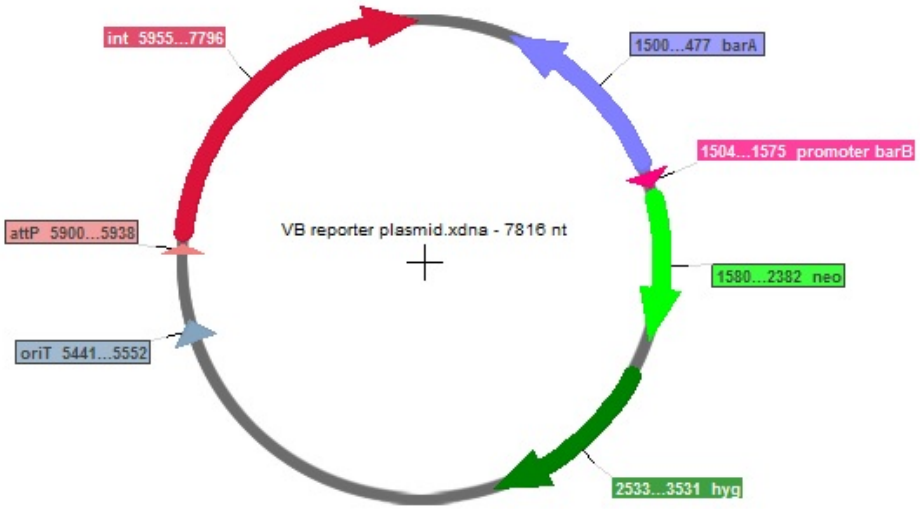
Figure 2.2: Schematic representation of pTE133-*arpA*-promoter *adpA* and pTE133-*barA*-promoter *barB* plasmids showing the orientation of (a) *arpA*, promoter-*adpA* (*adpA*p) and *neo* genes in the A-factor reporter construct and (b) *barA*, promoter-*barB* (*barA*p) and *neo* genes in the VB reporter construct. The HindIII and SacI rstriction sites used in cloning are also shown.

plasmids transformed into *E.coli* DH5$\alpha$. The plasmids were then introduced into the methylation-deficient *E.coli* strain ET12567 containing the RP4 derivative pUZ8002 and transferred to *S.coelicolor* LW16 by conjugation [14, 33]. Figure 2.3 gives a schematic maps of the final plasmids.

Single-crossover exconjugants were selected on SFM plates containing 50 $\mu$g/ml hygromycin and 25 $\mu$g/ml nalidixic acid, to obtain transconjugants LW16::pIJ82-*arpA*-promoter *adpA*-*neo* and LW16::pIJ82-*barA*-promoter *barB*-*neo*. The A-factor and VB reporter conjugants obtained were grown in ISP2 + 50 $\mu$g/ml hygromycin liquid medium for the DNA isolation and the plasmid integration was confirmed by PCR with primers attB-forw and attP-rev which gave an amplified product of 0.8 kb corresponding to the inserted region. Further, PCR with attB-forw, neo-rev primer pair was performed to amplify and confirm the sequence of the receptor, promoter and *neo* genes inserted in the cassette. The reporter strains were grown on SFM + 50 $\mu$g/ml hygromycin solid medium for the spore stock preparation. The harvested spores were grown in ISP2 + 50 $\mu$g/ml hygromycin liquid medium for the post-sporulation DNA isolation and analysis.




(a)




(b)

Figure 2.3: Schematic map of the A-factor and VB reporter plasmids prior to chromosomal integration. The figures (a) and (b) show the location of inserted genes in the A-factor reporter plasmid, pIJ82-*arpA*-promoter *adpA-neo*, and the VB reporter plasmid, pIJ82-*barA*-promoter *barB-neo*, respectively. The hygromycin resistance gene *hyg*, the selectable marker for the plasmids, and *int* gene (phiC31 integrase) for chromosomal integration of plasmids based on *attP* attachment site (phiC31 attP site) are also shown.

Table 2.1: Primers used in this study

| Primer/Oligonucleotide | Sequence (5'-3') |
|---|---|
| ArpA-SacI-forw | CTGAGAGCTCACGGTTTCCGGACAGACCGATG |
| ArpA-HindIII-rev | CTATAAGCTTCGTGATCCGCCATGTCCTC |
| BarA-SacI-forw | CTCAGAGCTCATAGAAACCGATCTCGCGGGAAC |
| BarA-HindIII-rev | CTCGAAGCTTGATACAAACCGTCAGACCCGATTGC |
| AdpA-SacI-forw | CAGTGAGCTCGGATACAGCACACGCAACCAATG |
| AdpA-SacI-rev | CTATGAGCTCGTTCGCGTCTCCCTGGTCCTTAC |
| Prom-BarB-rev | CTCGGTTTGCTGAACGTCTC |
| attB-forw | TTCTGGAAATCCTCGAAGGC |
| attP-rev | GTAAGCACCCGCGTACGTGT |
| neo-rev | TGTCTGTTGTGCCCAGTCAT |
| Prom-BarB-for (oligo) | CGCAGCACACGAGACGTTCAGCAAACCGAGCG-GTTCGCTTGCCTCAGCCAAACGGTG-CACGTCAGGAGTTGGAGCT |
| Prom-BarB-rev (oligo) | CCAACTCCTGACGTGCACCGTTTGGCTGAG-GCAAGCGAACCGCTCGGTTTGCT-GAACGTCTCGTGTGCTGCGAGCT |

Table 2.2: Strains and plasmids used in this study

| Strain/Plasmid | Genotype/Characteristics | Reference/Source |
|---|---|---|
| *S.coelicolor* strain M145 | Wild type *S.coelicolor* strain capable of producing SCBs | Kindly provided by Dr. Eriko Takano at University of Groningen |
| *S.coelicolor* strain LW16 | *scbA scbR* double deletion mutant | Hsiao *et al.* [23]. Kindly provided by Dr. Eriko Takano at University of Groningen |

Table 2.2: Strains and plasmids used in this study (contd.)

| Strain/Plasmid | Genotype/Characteristics | Reference/Source |
|---|---|---|
| *S.coelicolor* strain LW16::pTE134 | SCB reporter used in kanamycin resistance bioassay | Hsiao *et al.* [23]. Kindly provided by Dr. Eriko Takano at University of Groningen |
| *E.coli* ET12567/pUZ8002 | Methylation deficient *E.coli* ET12567 carrying the non-transmissible plasmid pUZ8002 containing *oriT* from the IncP-group plasmid RP4. Used for site-specific chromosomal integration of plasmid DNA through conjugation into *Streptomyces* | Flett *et al.* [14]. Kindly provided by Dr. Eriko Takano at University of Groningen |
| *E.coli* DH5$\alpha$ | Host for cloning: F- 80d*lacZ* M15 (*lacZYA-argF*) U169 *recA1* *endA1hsdR*17(rk-, mk+) *phoAsupE*44 -thi-1 *gyrA96 relA1* | Kindly provided by Dr. Micah Gearhart at the University of Minnesota |
| *E.coli* DH5$\alpha$:pTE133 | Plasmid pTE133 transformed into *E.coli* DH5$\alpha$ | This study |
| *E.coli* DH5$\alpha$:pIJ82 | Plasmid pIJ82 transformed into *E.coli* DH5$\alpha$ | This study |

Table 2.2: Strains and plasmids used in this study (contd.)

| Strain/Plasmid | Genotype/Characteristics | Reference/Source |
|---|---|---|
| *S.coelicolor* strain LW16::pIJ82-*arpA*-promoter *adpA-neo* | A-factor reporter | This study |
| *S.coelicolor* strain LW16::pIJ82-*barA*-promoter *barB-neo* | VB reporter | This study |
| *S.virginiae* | Wild type capable of producing virginiae butanolides | Kindly provided by Dr. Eriko Takano at University of Groningen |
| *S.griseus* | Wild type capable of producing A-factor | Kindly provided by Dr. Eriko Takano at University of Groningen |
| pGEM-T Easy | High-copy-number vector linearized with poly-T tail, containing the $\alpha$-peptide coding region of the enzyme $\beta$-galactosidase. Insertional inactivation of the $\alpha$-peptide allows identification of recombinants by blue/white screening on indicator plates | From Promega, Madison, WI |

Table 2.2: Strains and plasmids used in this study (contd.)

| Strain/Plasmid | Genotype/Characteristics | Reference/Source |
|---|---|---|
| pGEM-T-*arpA* | *arpA* in vector | This study |
| pGEM-T-promoter *adpA* | promoter *adpA* in vector | This study |
| pGEM-T-*barA* | *barA* in vector | This study |
| pTE133 | Plasmid carrying the promoterless kanamycin gene, *neo*, $Amp^R$ | Hsiao *et al.* [23]. Kindly provided by Dr. Eriko Takano at University of Groningen |
| pTE133-*arpA* | Plasmid with *arpA*-*neo* casette, $Amp^R$ | This study |
| pTE133-*barA* | Plasmid with *barA*-*neo* casette, $Amp^R$ | This study |
| pTE133-*arpA*-promoter *adpA* | Plasmid with *arpA*-promoter *adpA*-*neo* casette, $Amp^R$ | This study |
| pTE133-*barA*-promoter *barB* | Plasmid with *barA*-promoter *barB*-*neo* casette, $Amp^R$ | This study |

Table 2.2: Strains and plasmids used in this study (contd.)

| Strain/Plasmid | Genotype/Characteristics | Reference/Source |
|---|---|---|
| pIJ82 | 5.9 kb integrating vector containing phiC31 *attP* site, hyg$^R$ | Hsiao *et al.* [23]. Kindly provided by Dr. Eriko Takano at University of Groningen |
| pIJ82-*arpA*-promoter *adpA-neo* | Integrating vector with *arpA*-promoter *adpA-neo* , hyg$^R$ | This study |
| pIJ82-*barA*-promoter *barB-neo* | Integrating vector with *barA*-promoter *barB-neo*, hyg$^R$ | This study |

## 2.4 Kanamycin Resistance Bioassay

The published protocol for the kanamycin resistance bioassay with the SCB reporter was optimized for our laboratory conditions. The concentration of kanamycin and pH of the medium were optimized for the clear visualization of positive results. This was achieved by performing the assays in media prepared with kanamycin concentrations varying from 3 - 7.5 $\mu$g/ml and the pH adjusted from 6.3 - 6.8 by addition of 100 mM HCl, while keeping the amount of SCB extract constant. The crude SCB extract from *S.coelicolor* M145 was prepared following the method given by Hsiao *et al.* to use as a positive control for the kanamycin resistance bioassay [22]. The M145 strain was grown on SMMS medium for 5 days and extracted using ethyl acetate. The ethyl acetate was removed with rotary evaporator and the SCB extract re-suspended in 100% HPLC-grade methanol at a concentration of approximately 10 $\mu$l per plate extracted. The extract was tested for positive activity by spotting on Difco Nutrient Agar (DNAgar) plates containing 3 - 5 $\mu$g/ml kanamycin which was spread with SCB reporter spores at

a concentration of $2\times10^6$ spores in 100 $\mu$l volume. Methanol was used as the negative control. A positive result was identified by the appearance of kanamycin resistant colonies around the spot after incubation at 30°C for 48 hrs. Absence of any growth around the spot (except some spontaneous mutants randomly dispersed on the plate) indicated a negative result.

### 2.4.1 Screening extract libraries with the SCB and A-factor reporters

A pilot extract library was created by extracting seven isolates from a *Streptomyces* sp. strain collection and *S.griseus* according to the rapid GBL extraction protocol (as described in the previous section). The strains were grown on SMMS medium for 3 days at 30°C and extracted with ethyl acetate. All extracts were tested with the SCB and A-factor reporters in the kanamycin resistance bioassay using the previously described protocol.

The SCB and A-factor reporters were also screened with 37 extracts from a *Streptomyces* liquid culture extract library. Extracts used in the screening were prepared from liquid cultures grown for 5 days in ISP2 broth with shaking and extracted with ethyl acetate. The solvent phases were dried using a rotary evaporator and reconstituted in methanol at a standard concentration (10 mg/mL).

### 2.4.2 Modified kanamycin resistance bioassay: overlay method

In order to circumvent the lengthy GBL extraction process and increase the throughput, the protocol for the kanamycin resistance bioassay was modified to enable testing of live *Streptomyces* cultures with the GBL reporters. The strains to be tested with the reporters were grown on solid ISP2 medium at 30°C for 1-3 days. At the end of the growing period, cultures were overlaid with 10 ml of 1% nutrient agar containing 4.5 $\mu$g/ml kanamycin (pH 6.4 unadjusted), seeded with approximately $2 \times 10^5$ cfu/ml of the reporter spores. These plates were then incubated at 30°C for 48 hours. A non-inoculated ISP2 plate was also overlaid to serve as a negative control for the assay. The plates were observed at the end of the incubation period for the measurement of growth of the reporter around the *Streptomyces* test colonies.

**Screening a *Streptomyces* collection with A-factor and SCB reporters in the overlay assay**

The overlay method was utilized to screen a collection of 40 *Streptomyces* isolates with the A-factor and SCB reporters for investigating signaling activity. The isolates were selected for screening from a *Streptomyces* strain collection based on their ability to grow well on the ISP2 medium at 30°C. In all cases, 2 $\mu$l of the frozen spore stocks were spotted on the ISP2 plate for starting the culture. The strains isolated from a variety of environments were represented in the collection. To account for the potential chemical instability of signaling molecules in the ISP2 medium as well as the temporal variability in signal production, 1, 2 and 3 day cultures of each isolate was tested with the reporters.

Since many *Streptomyces* isolates are known to produce antibiotic-modifying or degradative enzymes, further control experiments were designed to monitor and inactivate any kanamycin degrading activity of the test isolates that could interfere with the kanamycin resistance overlay bioassay. To detect the kanamycin degrading activity, the reporter spores were replaced with *S.coelicolor* strain LW16 spores under identical conditions in the kanamycin resistance overlay assay of the test isolates. Additionally, the kanamycin resistance overlay protocol was modified to include the exposure of the *Streptomyces* test cultures to chloroform in order to eliminate the effect of kanamycin modifying enzymes produced by the test isolates. Filter paper soaked with approximately 3 ml chloroform per plate was placed inside the lid of glass petri dishes containing the growing isolates. The plates were kept inverted in the fume hood for 3 hours allowing the chloroform vapors to saturate the strains. The chloroform vapors were removed by leaving the plates open in a fume hood for 1 hour. The cultures were then overlaid with the A-factor and SCB reporters and the *S.coelicolor* LW16 control strain in the kanamycin resistance overlay assay.

### 2.4.3 Liquid culture assay

Preliminary liquid culture studies were performed with the reporters in an attempt to develop a liquid-based assay for high-throughput screening. In 200 ml erlenmeyer flasks containing 50 ml YEME medium, $1\times10^5$ cfu/ml reporter spores were grown with

4.5 $\mu$g/ml kanamycin and 0.1 $\mu$l/ml microbial extract. Separate control experiments were performed without addition of kanamycin and microbial extracts to the medium. All flasks were incubated at 30°C with shaking at 250 rpm. At the end of the incubation period, optical density of cultures was measured using a spectrophotometer. The liquid culture volume was also reduced to 200 $\mu$l to conduct the assay in the 96-well format, keeping the other parameters identical. The plates were incubated with shaking at 250 rpm and the OD600 was measured using a plate reader.

# Chapter 3

# Results and Discussion

## 3.1 Genetic Analysis of A-factor and VB Reporters

A-factor and VB reporters were assembled successfully following the published protocol for the SCB reporter system [23]. Each step of the DNA recombination was accompanied by PCR amplification and DNA sequencing of the inserted genes using appropriate primers in order to ensure the intact and error free insertion of genes in the selected clones.

To test the genetic stability of the reporter stains, a comparative PCR analyis of mycelial genomic DNA before and after sporulation was performed. The PCR with the attB-forw, attP-rev primer pair was used to confirm chromosomal integration based on attachment sites and the PCR using the attB-forw, neo-rev primer pair was used to amplify the region from the attachment site in the genome to the receptor, promoter and *neo* genes inserted in the cassette. The agarose gel electrophoresis analysis of these PCR product bands showed that the constructs were genetically unstable and underwent spontaneous insertion and deletion during the process of sporulation (Figure 3.1). HindIII restriction digestion of the purified PCR product further confirmed the occurrence of these mutations in DNA isolated post sporulation. Figure 3.1 a gives a schematic of the orientation of genes after chromosomal integration and the primers used in PCR. The agarose gel images in Figure 3.1 b and 3.1 c show the effect of mutation on DNA fragment size.

In order to address the genetic instability, selective pressure in the SFM medium

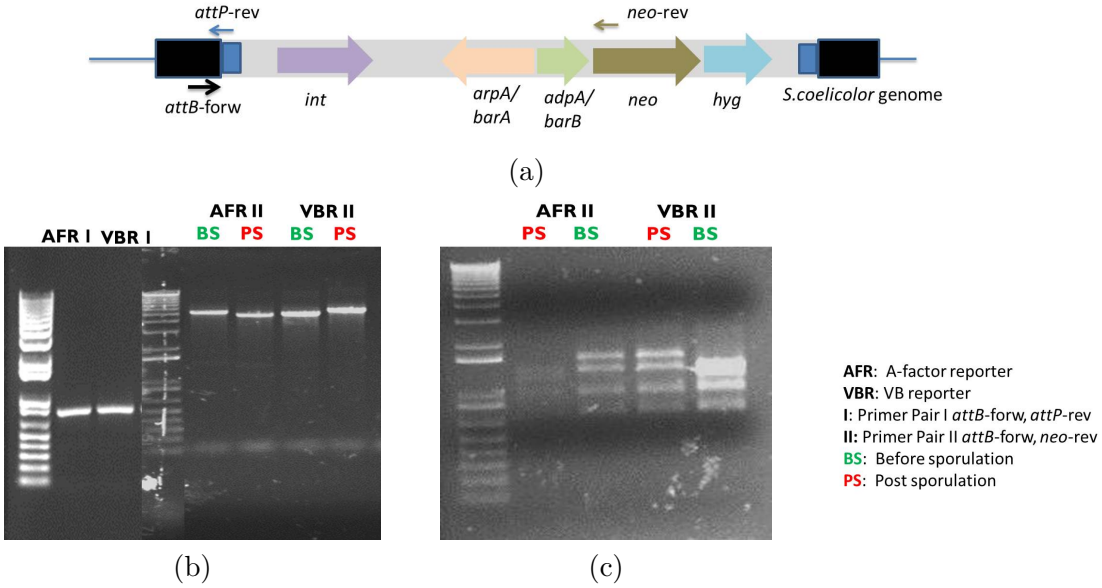



Figure 3.1: Agarose electrophoresis analysis of the A-factor and VB reporter genomes for inserted genes. The image (a) is a schematic of the *S.coelicolor* genomic DNA showing the expected orientation of genes after integration. The arrows indicate the location of primers that were used in PCR to confirm the integration. The images (b) and (c) are the agarose gels of PCR products and restriction digests on the A-factor and VB reporter (AFR and VBR respectively) genomic DNA showing the effect of spontaneous mutation in post-sporulated DNA. The PCR with the attB-forw, attP-rev (primer pair I) was used to confirm chromosomal integration and gives a 0.8 kb product with both A-factor and VB reporters (AFR I and VBR I in the image). The PCR with the attB-forw, neo-rev (primer pair II, AFR II and VBR II in the image) forms a product spanning the chromosomal attachment site and the inserted genes. The expected size of this PCR product is approx. 3.9 kb for the AFR and 3.5 kb in the case of the VBR. The image (b) confirms successful chromosomal integration and also shows a clear difference in the PCR product size from amplification of the A-factor and VB reporter DNA with the attB-forw, neo-rev primer pair before sporulation (BS) and post sporulation (PS). The gel image (c) is the HindIII restriction digest of this product and depicts the differences in the size of fragments before and after sporulation, further confirming the spontaneous insertion and deletions in this region.
(b) lanes: 1- DNA ladder, 2- Primer pair I PCR of AFR PS (AFR I) 3- Primer pair I PCR of VBR PS (VBR I), 4- DNA ladder, 5- Primer pair II PCR of AFR BS (AFR II BS), 6- Primer pair II PCR of AFR PS (AFR II PS), 7- Primer pair II PCR of VBR BS (VBR II BS), 8- Primer pair II PCR of VBR PS (VBR II PS);
(c) lanes: 1- DNA ladder, HindIII digest of the primer pair II PCR product of 1- AFR PS (AFR II PS), 2- AFR BS (AFR II BS), 3- VBR PS (VBR II PS), 4- VBR BS (VBR II BS);

was increased to 75 $\mu$g/ml hygromycin while growing the A-factor and VB reporters for the spore stock preparation. The PCR analysis showed that this stabilized the A-factor reporter, but failed to stabilize the VB reporter (see Figure 3.2). The orientation and integrity of all the inserted genes in the A-factor reporter was confirmed by sequencing the purified product from the attB-forw, neo-rev primer pair PCR reaction with primers for *arpA*, promoter *adpA* and *neo* genes. The selective pressure in the medium for the spore stock preparation was further increased to 100 $\mu$g/ml hygromycin in an effort to stabilize the VB reporter spores. However, it was observed that the inserted genes were still unable to be amplified from the mycelial DNA post sporulation. Since the VB reporter spores could not be stabilized, only the A-factor reporter spores were analyzed for functionality using the kanamycin resistance bioassay.

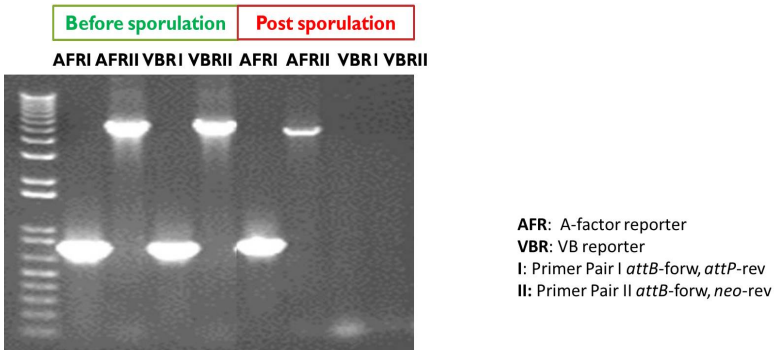
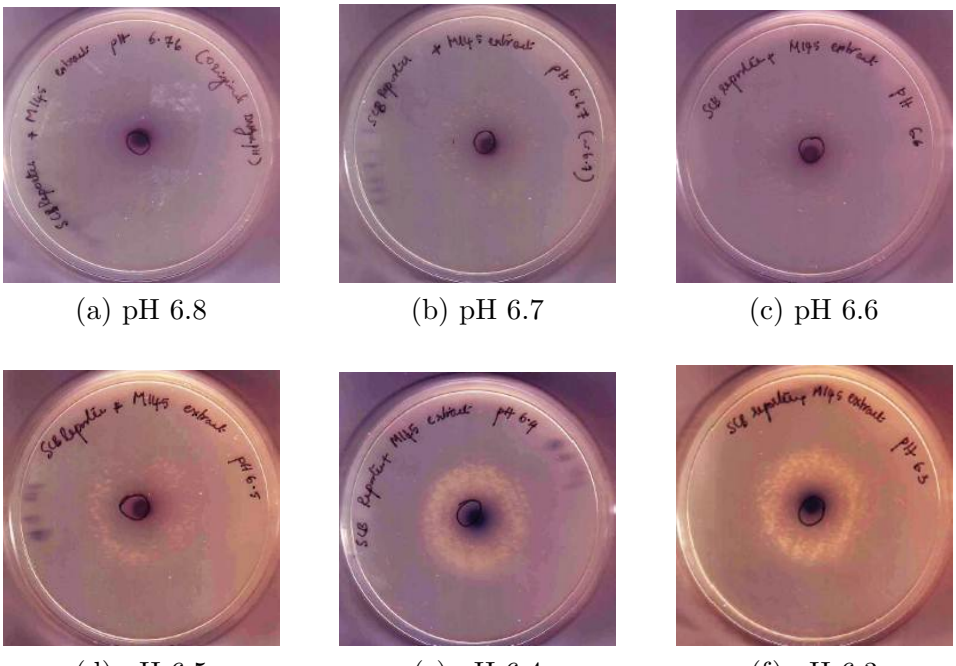



Figure 3.2: The effect of increased hygromycin selection on the A-factor and VB reporter DNA. The gel image demonstrates the stabilization of the A-factor reporter (AFR) spores after increasing hygromycin selective pressure to 75 $\mu$g/ml and illustrates its lack of effect in the case of the VB reporter (VBR). The genomic DNA extracted before and after the sporulation was PCR amplified with attB-forw, attP-rev (primer pair I, expected product size 0.8 kb) and attB-forw, neo-rev (primer pair II, expected product size 3.9 kb for AFR and 3.5 kb for VBR) primers and compared. Lanes are: 1- DNA ladder, 2- Primer pair I PCR of AFR Before Sporulation (BS), 3- Primer pair II of AFR BS, 4- Primer pair I PCR of AFR Post Sporulation (PS), 5- Primer pair II PCR of AFR PS, 6- Primer pair I PCR of VBR BS, 7- Primer pair II PCR of VBR BS, 8- Primer pair I PCR of VBR PS(no product), 9- Primer pair II PCR of VBR PS (no product).

## 3.2 Bioassays with the A-factor and SCB Reporters

### 3.2.1 Effect of pH on the reporters

It was observed that the pH of the commercial Difco nutrient agar media used in the kanamycin resistance bioassay varied from pH 6.4 to 6.8 and this had a significant effect on the response of the SCB reporter. Testing the SCB reporter with identical amounts of M145 extract on media of pH ranging from 6.8 - 6.3 (see Figure 3.3) showed most intense response with minimal background growth at pH 6.4. The growth of background mutants increased considerably at pH 6.3 suggesting that the lower pH affected the stability and thereby the potency of kanamycin in the medium.



(a) pH 6.8 (b) pH 6.7 (c) pH 6.6

(d) pH 6.5 (e) pH 6.4 (f) pH 6.3

Figure 3.3: The effect of the pH of the medium on the response of the SCB reporter to identical amounts of positive control M145 extract. All plates were prepared with Difco Nutreint Agar and kanamycin (4.5 $\mu$g/ml). The reporter growth halo starts appearing at pH 6.5 and showed the most robust growth with minimal background at pH 6.4. Lower pH values led to both increased growth of the reporter and larger number of random mutants (observed as the growth in areas away from where the extract was spotted). (Note: With higher amount of M145 extract, growth of the SCB reporter can be observed at higher pH.)

The pH of the medium was found to have an effect on the A-factor reporter as well.

As observed in the case of the SCB reporter, lower pH levels corresponded to higher levels of background growth of the reporter in the absence of any signaling molecule (data not shown). At higher pH, lower kanamycin concentrations resulted in fewer mutants while at lower pH, higher kanamycin concentration was required. In contrast with the SCB reporter conditions, at 4.5 $\mu$g/ml kanamycin and pH 6.4, the A-factor reporter exhibited significant background and therefore for testing the A-factor reporter, the optimal pH was found to be pH 6.8.

### 3.2.2 Kanamycin resistance overlay bioassay

**Testing the A-factor reporter**

To positively confirm the activity of the A-factor reporter, it has to be tested with A-factor in the kanamycin resistance bioassay. The extraction of A-factor from the producing organism, *S.griseus*, was attempted for this purpose. Since conventional large-scale liquid culture extraction was not feasible, *S.griseus* solid culture crude extract was prepared following a published method [22]. Different media conditions were tested for successfully extracting A-factor from solid culture. *S.griseus* was grown in both complex medium (nutrient agar) and minimal medium (SMMS) and the cultures were extracted at different time points (24 hrs, 48 hrs and 72 hrs). All extracts failed to elicit positive response when tested with the A-factor reporter.

The lack of standard signal compounds and a reliable method for isolation of A-factor required development of an alternative method to test the A-factor reporter. An overlay bioassay was subsequently designed to test the live culture of *S.griseus* directly with the A-factor reporter in the kanamycin resistance bioassay. Since A-factor is produced in a time sensitive manner and might not be stable in complex growth media such as ISP2, a time course experiment was designed. The *S.griseus* cultures were grown for 1, 2, 3, 4 and 5 day time points and tested with the A-factor reporter. The A-factor reporter was observed to grow around the *S.griseus* colony at all time points tested as shown in the Figure 3.4. These results initially suggested that A-factor was produced by *S.griseus* starting from day 1 and the A-factor reporter was able to detect it in the kanamycin resistance overlay bioassay. Similar results were observed when the SCB reporter was tested in the kanamycin resistance overlay bioassay with *S.coelicolor* M145 strain (data

not shown).

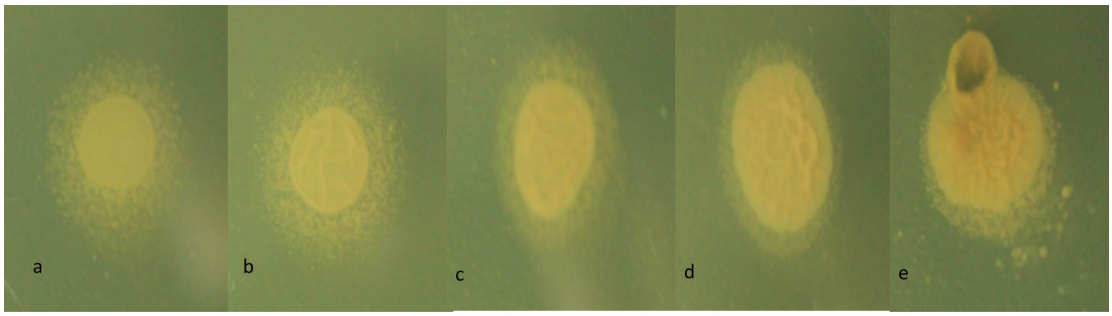


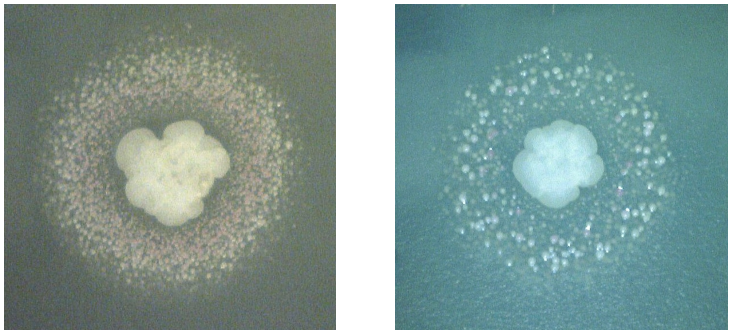

Figure 3.4: Results from the kanamycin resistance overlay assay of *S.grisues* with the A-factor reporter. The images (a - e) show 1 - 5 day *S.griseus* cultures overlaid with the reporter in media containing kanamycin (4.5 $\mu$g/ml). The growth halo appear to be most dense around 1 day and 2 day *S.griseus* cultures and decrease thereafter.

### Screening a *Streptomyces* strain collection with the A-factor and SCB reporters using the kanamycin resistance overlay method

The apparent ease at which the kanamycin resistance overlay assay can be performed with the A-factor and SCB reporters led to the screening of 39 *Streptomyces* strains from a *Streptomyces* strain collection and the VB producing strain, *S.virginiae*, with the reporters. *S.virginiae* did not activate either of the reporters. Twenty out of the 39 strains (51%) from the strain collection were able to activate either the A-factor reporter or the SCB reporter on one or more time points. In most cases the same isolates activated both the reporters, but exceptions were also observed. Some strains exclusively activated either the A-factor reporter or the the SCB reporter. It was also found that in the isolates which activated both the reporters, activation of one reporter coincided temporally with the activation of the other reporter. Though this could be considered an indication that the GBL-like molecules produced by these strains were able to bind to both the A-factor and SCB receptors, a further control experiment was performed to verify that the observed results were true positives.

Earlier results on the effect of pH of the medium on kanamycin and the subsequent effect this has on the response of the reporter strains in the kanamycin resistance bioassay pointed to the possibility that factors affecting kanamycin might be present in the overlay bioassay. In order to test this hypothesis, all the strains which caused reporter

growth at 2 day time points in the kanamycin resistance overlay bioassay were selected and the assay repeated using *S.coelicolor* LW16 strain instead of the reporter strains. *S.coelicolor* LW16 strain lacks the kanamycin resistance gene and cannot grow in the presence of kanamycin. It is physiologically similar to the reporter strains in all other aspects and thus it is an ideal control strain for testing the presence of factors that could be compromising the overlay bioassay. Including *S.coelicolor* and *S.griseus*, 15 strains were tested with the LW16 strain in the kanamycin resistance overlay control experiment. It was observed that with the exception of one strain (CES-233), all tested strains caused LW16 to form a growth halo similar to those formed by the reporters (see Figure 3.5 for a sample comparison of the growth of LW16 and the A-factor reporter caused by a test strain).



(a) (b)

Figure 3.5: Comparison of the A-factor and the strain LW16 growth halo around a test strain, *Streptomyces* sp. CES-311. The images (a) and (b) show the growth of the A-factor reporter and LW16 strain around a 2 day colony of CES-311 in the kanamycin resistance overlay bioassay under identical conditions. The growth halo appear to be denser around the reporter compared to strain LW16.

The results showed that the growth of the reporters around the colonies of tested strains were not caused by the signal detection and the subsequent kanamycin resistance induction, but due to the tested strains themselves modifying kanamycin in the surrounding medium. Kanamycin is an aminoglycoside antibiotic and enzymatic modification is the most common type of aminoglycoside resistance mechanism [54]. Aminoglycoside modifying enzymes have been reported from several *Streptomyces* sp. including *S.griseus* [54]. The results of the control experiment suggested that kanamycin modifying enzymes are widespread among *Streptomyces* and caused interference to the signal

reporting process by the GBL reporters.

**Chloroform treatment of *Streptomyces* cultures in the overlay assay**

In spite of maintaining identical assay conditions and spore density in the overlay, differences were observed in the density of the halo produced by the reporter strains and LW16 around the test strains. As seen in Figure 3.5 a and 3.5 b, the halo formed by the A-factor reporter was significantly denser than LW16 strain. This pointed towards the possibility of a combination effect in which kanamycin in the medium is inactivated due to the combined activity of kanamycin degradation by the test strain and the signal detection by the reporter strain. To test this hypothesis, the overlay protocol was modified to include a 2 hr exposure of the test strains to chloroform before overlaying with the reporters and LW16. The exposure to chloroform is a widely utilized technique in overlay bioassays to kill test strains prior to overlay. All strains that caused reporter growth at all time points were re-tested with the A-factor reporter, SCB reporter and LW16 strain using the modified overlay protocol. The results from chloroform treatment are compiled in Table 3.1. The exposure to chloroform prevented the growth halo by both the reporters and LW16 in nine out of 22 strains tested. This included the positive control strains, *S.coelicolor* M145 and *S.griseus*. Among the 13 test strains which caused the growth of either of the reporters, nine strains clearly caused the growth of LW16 as well.

Although there appeared to be variations in the densities of halo produced by LW16 and the reporters, it was not sufficient to differentiate between true signal detection and false positives. It was concluded that the variations observed in the density of halos produced by the test strains with the SCB reporter, A-factor reporter and LW16 in the overlay bioassay were artifacts caused by variations in spore density of the stocks, amount of overlaid kanamycin agar, etc. The reporters appeared to grow in very low numbers around the remaining four strains and were scored as weakly positive. The overall results showed that chloroform treatment did not sufficiently inhibit the kanamycin degrading or modifying enzymes among the *Streptomyces* stains tested. Additionally, the inability of even the positive control strain, *S.coelicolor* M145 to induce the SCB reporter after chloroform treatment indicated that live *Streptomyces* cultures do not produce sufficient amounts of signaling molecules to be clearly detected using this method.

Therefore, additional screenings were focused on concentrated extracts from cultures.

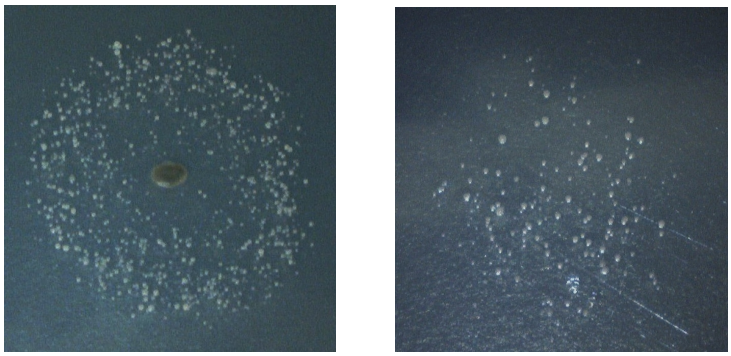
| Treatment | Number of strains that caused the reporter growth in the presence of kanamycin | Number of strains that caused the growth of strain LW16 in the presence of kanamycin |
|---|---|---|
| Without chloroform exposure | 22 out of 42 | 14 out of 15 |
| With chloroform exposure | 13 out of 22 | 9 out of 22 |

Table 3.1: Results from testing the strain collection (including *S.coelicolor* M145, *S.griseus* and *S.virginiae*) with the A-factor and SCB reporters and strain LW16 in the kanamycin resistance overlay bioassay, showing the effect of chloroform treatment. The table shows the number of strains that caused either of the reporters to grow in the presence of kanamycin out of the total number of strains tested, with and without chloroform treatment. The total number of strains tested with the negative control strain LW16 and the number of strains that caused LW16 growth in kanamycin medium is also given (Note: Only 15 out of 22 strains that caused the growth of the reporters were tested with LW16 strain in the assay without chloroform treatment).

### 3.2.3 Screening of a *Streptomyces* extract library with the SCB and A-factor reporters

Two crude extract libraries were tested with the A-factor and SCB reporters to screen for signaling activity. A small extract library was prepared from 3 day minimal media solid cultures of seven strains from *Streptomyces* strain collection as described in Hsiao *et al.* for testing with the SCB reporter [23]. Out of seven extracts, the extract of strain CES-233, an isolate from Mississippi river sediment, induced the growth of the SCB reporter (see Figure 3.6 a) in the kanamycin resistance bioassay, suggesting that CES-233 produces GBL like compounds capable of binding to the SCB receptor protein. The A-factor and SCB reporters were also used to screen a liquid culture extract library. Ethyl acetate extracts from 5-day liquid cultures of 37 *Streptomyces* strains were screened with the reporters in the kanamycin resistance bioassay. The extract from a Florida marine sediment isolate CES-015 was the sole sample which induced the SCB reporter (see Figure 3.6 b).

Neither of the two extracts that caused the growth of the SCB reporter induced



(a) (b)

Figure 3.6: Induction of the SCB reporter by extracts of *Streptomyces* isolates (a) CES-233 (solid culture extract) and (b) CES-015 (liquid culture extract) in the kanamycin resistance bioassay performed under identical conditions. The significantly larger halo observed with CES-233 solid culture extract could be due to higher concentration of the extract or more specific binding of reporter inducing compounds compared to CES-015 liquid culture extract.

similar response in the A-factor reporter, suggesting that either the observed results are specific to the SCB reporter. However, it could also be indicative of the inability of A-factor reporter assay to detect signaling molecules. The significantly larger halo observed with the CES-233 solid culture extract could be indicative of either higher concentration or higher binding affinity of the SCB receptor binding compounds in the extract compared to CES-015 extract. Both the strains have been previously characterized for antibacterial and anti-fungal activities and CES-015 is known to exhibit antibacterial and anti-fungal activity whereas CES-233 is active against Gram positive bacteria (unpublished data). The role of the SCB reporter inducing compound in this observed antibiotic and anti-fungal activity or other hitherto unknown behaviors in these strains is a topic for further investigation.

The results from the screening of the extract library points to several complexities associated with the search of GBL molecules. The SCB reporter was induced by merely 4.5% (2 out of 44) of the tested samples, while the A-factor reporter was induced by none. The absence of reporter induction by a majority of the extracts, most of which have been previously shown to have significant bioactivity against bacteria and fungi and are good candidates for potential signaling activity, could be due to several reasons. First, the strains from which the extracts were prepared might not be producing signaling

molecules capable of binding to the GBL receptor proteins in the reporters. Other possibilities include the non optimal culture conditions, medium components, method of extraction and concentration of extracts. All extracts in the extract library were prepared from small scale cultures. While small scale solid culture extraction has been proven to be successful for *S.coelicolor* GBLs, it failed to produce an active extract in the case of *S.griseus*. For GBL extraction from liquid cultures, large volumes in the range of hundreds of liters are typically required, whereas the liquid culture library used in this screening was prepared from much smaller volumes. The results observed could be an indication of either the A-factor reporter not detecting A-factor successfully or none of the tested extracts containing A-factor like GBLs. It should be noted that A-factor is the only known GBL of its type, so A-factor type GBLs might be less commonly produced compared to SCB type.

### 3.2.4 Liquid culture studies with the SCB reporter

In order to expand the kanamycin resistance assay into a robust, high throughput format, preliminary liquid culture studies were performed with the SCB reporter. The SCB reporter was grown in liquid culture under various conditions and it was found that the reporter was successfully induced in liquid culture in the presence of kanamycin when the positive control *S.coelicolor* M145 extract was added. The growth of the SCB reporter was quantified using optical density measurements (Table 3.2).

| Sample | Calculated OD |
|---|---|
| Positive control: SCB reporter | 2.682 |
| Negative control: SCB reporter + kanamycin | 0.000 |
| SCB reporter + kanamycin + M145 extract | 1.848 |

Table 3.2: The optical density of SCB reporter grown in liquid culture under various conditions. The culture conditions were maintained identical in all other respects. The optical density measurement at the end of the culture period showed that kanamycin resistance in the SCB reporter was successfully induced in the liquid culture when *S.coelicolor* M145 extract was added.

Subsequently, the SCB reporter assay was conducted in a 96-well plate high throughput liquid culture screening format. However, when scaled down to the 200 $\mu$L assay

volume in the 96-well plates, the optical density measurement at the end of the incubation showed that the SCB reporter failed to grow in the wells even in the absence of kanamycin in the medium. Although other assay conditions were identical to flask cultures, 96-well plates have limitations in aeration and mixing which presumably affected the ability of the reporter strain to grow.

# Chapter 4

# Conclusions

*Streptomyces* is a genus of considerable interest since they are the most important source of antibiotics for medical, veterinary and agricultural use [9]. Study of signaling among *Streptomyces* has potential applications in various fields like drug discovery, ecology and agriculture. The extent of signaling among *Streptomyces* in terms of frequency and specificity, the identity of various types of signaling molecules, their roles in inter-species and possibly inter-kingdom interactions through regulation of important processes like antibiotic production is a growing field of study. Practical applications of this field include the potential to harness signaling molecules to induce or up-regulate the production of novel or important secondary metabolites like antibiotics or anti-cancer compounds from *Streptomyces*. The largest class of signaling molecules that have been identified from *Streptomyces* are GBLs. In this study, the development of two new GBL reporters was undertaken and screening studies were conducted together with a previously developed reporter to expand our knowledge of the GBL signaling process.

The induction of antibiotic production is the traditional approach used for detecting GBL signaling molecules produced by *Streptomyces*, but this method has several drawbacks. In co-cultures it can not give clear results if both strains are capable of producing antibiotics, as is often the case with *Streptomyces*. It also does not provide information about the potential chemical identity of the signaling molecule or the ligand specificity to the receptor. Therefore, receptor based reporters are highly desirable and such a system for detecting A-factor and VB type GBLs was attempted in this study. In conjunction with the previously developed SCB reporter, these reporters serve as tools for studying

GBL mediated signaling among *Streptomyces*. By screening *Streptomyces* extract libraries using the A-factor and SCB reporters in the kanamycin resistance bioassay, two environmental isolates from diverse geographical locations with known antibiotic activity were identified to produce GBLs, most likely SCB type. The isolation, structural elucidation and functional role determination of these potentially new GBL type signaling compounds from the isolates will aid in expanding our knowledge of GBL based signaling systems.

The results from the screening showed that the proper culture and extraction techniques are key to the successful identification of GBL-like compounds from *Streptomyces* using the reporters. The extracts prepared from three day cultures grown on solid minimal medium consistently showed a higher likelihood for the presence of GBL-like compounds. In this study, one out of seven environmental *Streptomyces* isolate cultures extracted using this method induced the SCB reporter. This is similar to the results of Hsiao *et al.* where identically prepared extracts from two out of nine known antibiotic producing *Streptomyces* strains induced the SCB reporter. In contrast, only one out 37 extracts from the liquid culture extract library was able to induce the SCB reporter.

An attempt was made to modify the assay in order to circumvent the lengthy GBL extraction process. This was done by testing live cultures of *Streptomyces* with the SCB and A-factor reporters in the modified kanamycin resistance overlay bioassay. Multiple time points were used in the assay to account for the temporal variability in the GBL production and the chemical instability of GBLs in the medium. The relative ease with which this assay could be performed enabled a large number of strains to be screened with the GBL reporters at different growth stages in this study. Although the initial results from the kanamycin resistance overlay bioassay were promising, performing further control experiments showed that kanamycin resistance mechanisms are widespread among soil *Streptomyces* and this caused interference with kanamycin resistance reporter gene in the reporters. A high throughput liquid culture format for the kanamycin resistance bioassay was also attempted. Though the initial large-scale liquid culture study was successful, 96-well plate culture conditions were found to require further optimization.

## Future Directions

Phylogenetic analysis of GBL synthases has shown that the A-factor synthase, AfsA, shares the highest amino acid identity (66%) to the SCB synthase, ScbA [57]. However, none of the extracts tested in this study, including two strains that induced the SCB reporter, activated the A-factor reporter. While this could be an indication that A-factor type GBLs are less common than SCB type, it pointed to the necessity for a synthetic, pure A-factor standard to measure its sensitivity and to positively confirm its ability to bind to A-factor. A-factor was successfully synthesized after this study was concluded and when tested with the A-factor reporter, it failed to elicit a positive response (unpublished data). The root cause for the inability of the reporter to detect the signaling molecule successfully will need to be investigated and based on that necessary modifications have to be implemented in the A-factor reporter. New strategies to address the instability of the VB reporter also need to be developed and its ability to bind to VB molecules positively confirmed so that the VB reporter can be used in conjunction with the A-factor and SCB reporters for studying VB-type GBLs in *Streptomyces*.

The clear logical extension of the screening study would be to understand the chemical identity of molecules that induced the SCB reporter in the kanamycin resistance bioassay. Larger scale culture extractions followed by techniques such as bioassay guided fractionation using HPLC and structure determination using NMR may be employed to identify the active GBL type compounds from the extract. Further cell culture studies and gene expression analysis are required for identifying the role of these GBLs in inducing the antibiotic production or other behaviors in the producing species. Many of the *Streptomyces* strains in the strain library which are known to have antibacterial and anti-fungal activities, but did not induce the reporters in this screening study need to be properly extracted and screened again with multiple reporters for the presence of GBL type compounds.

The results of this study show that it is necessary for microbial cultures to be extracted in a specific extraction process for the GBLs to be detected by the reporters. This could potentially impede the use of reporters in large-scale study of ecological interactions among *Streptomyces* through GBL signaling. Other issues observed with

this reporter system are the genetic instability of the *Streptomyces* chromosomal constructs, the sensitivity of kanamycin to media conditions and the interference caused by kanamycin resistant *Streptomyces* sp. All these factors affect the robustness of the reporters and point to the need to explore additional strategies for developing GBL reporters. The substitution of the kanamycin resistance gene with other commonly utilized reporter genes such as green fluorescent protein (GFP) might prove beneficial, especially with regard to lowering the detection limit to enable testing of live cultures as well as high throughput liquid culture assays. Even though using *S.coelicolor* as the host strain for developing the reporters ensured that GBLs are properly transported into the cell, using alternative strategies like lysing the cells prior to testing could potentially enable other strains, like methylation deficient *E.coli* ET12567, to be used as host. An *E.coli* based reporter might prove to be more genetically stable, easily adaptable to high throughput 96-well plate formats and also shorten the assay length due to faster growth.

These strategies need to be explored for developing stable and easy to use GBL reporters that will function as valuable tools in furthering *Streptomyces* signaling research.

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