GENETIC MANIPULATION OF SECONDARY METABOLITE PRODUCTION IN ACTINOMYCETES

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TITLE: Genetic Manipulation of Secondary Metabolite Production in Actinomycetes

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Abstract

The world is facing a public health threat due to increasing emergence of antibiotic resistance in pathogens. *Streptomyces* the soil-dwelling, Gram-positive, filamentous bacteria belonging to the family actinomycetes, are proven to be rich sources of natural antibiotics. Genome sequencing of *Streptomyces coelicolor*, a model organism of this genus, has revealed that in addition to the five antibiotics characterized so far, it possesses abundant genetic architecture of unexpressed biosynthetic or cryptic clusters for secondary metabolite production. The reason for their silence appears to be the poor understanding of their specific activation stimuli. In *Streptomyces coelicolor*, a pleiotropic regulator belonging to the two-component system family, *afsQ1*, has shown to activate the production of actinorhodin (ACT), undecylprodigiosin (RED), and calcium-dependent antibiotic (CDA). The aim of this research was to employ the genetically engineered *afsQ1* allele (named *afsQ1*), which mimics the phosphorylated active form and obviates the need for specific external stimulus, and screen for novel antibiotic production. In this study, *afsQ1* was introduced in various wild actinomycete isolates from the Wright Actinomycetes Collection (WAC) by conjugation and the resulting mutants were screened for antibiotic production. Two out of six WAC strains showed *afsQ1*- induced antimicrobial activity. Interestingly, we were able to purify two antibiotic compounds, namely 1082 [M+2H]^{2+} and 782 [M+H]^+ from the strain WAC00263. 1082 [M+2H]^{2+}, a potentially novel antimicrobial peptide, exhibited activity against a wide range of Gram-positive bacteria including resistant pathogens such as vancomycin-resistant *Enterococcus* ATCC# 51299, a clinical isolate of methicillin
resistant *Staphylococcus aureus*, and a clinical isolate of *S. aureus* BM3002. Moreover, it also showed activity against an opportunistic Gram-negative multi-drug resistant pathogen *Acinetobacter baumannii* B0098426R and a virulent strain of the fungus *Cryptococcus neoformans* H99. The second newly expressed molecule, 782 [M+H]⁺ was not as potent as 1082 [M+2H]²⁺, so far only exhibited antimicrobial activity against the Gram-positive laboratory strains *Bacillus subtilis* #168 and *Micrococcus luteus*. These results reiterate that the technique of heterologous expression of the pleiotropic regulator, *afsQ1*, in diverse actinomycetes is an excellent tool to induce novel antimicrobial production.
To Anas and My Parents
Acknowledgements

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Abbreviations

ACT - Actinorhodin
ARC - Antibiotic Remodelling Compound
ATCC – American Type Culture Collection
CDA - Calcium Dependent Antibiotic
HPLC - High Performance Liquid Chromatography
LC-MS - Liquid Chromatography-Mass Spectrometry
MIC – Minimum Inhibitory Concentration
NMR - Nuclear Magnetic Resonance
RED - Undecylprodigiosin
TCS - Two Component Signal transduction system
WAC - Wright Actinomycetes Collection
Declaration of Academic Achievement

All the work in this thesis is completed by me with the exception of – Patricia Pak of the Elliot Lab created the plasmid pSET152-ermE*p-null, pSET152-ermE*p-afsQ1 and Daniel Socko from our Lab created the plasmid pSET152-ermE*p-afsQ1*; Primers used were designed by Tomas Gverzdys from our Lab. Tandem mass spectrometry (MS-MS) and accurate mass determination for the pure compound 1082 [M+2H]^{2+} was done by McMaster Regional Centre for Mass Spectrometry and Edman Degradation and Sequencing was conducted by Hospital for Sick Children, Toronto. The wild isolate actinomycetes (WAC) were collected by the Wright Lab.
Chapter 1: Introduction

1.1 Need for Discovery of Novel Antibiotics

Many bacteria synthesize secondary metabolites such as antibiotics as a strategy to deal with nutritional competition from neighbouring bacteria. These molecules can also inhibit other living organisms at much lower concentrations\(^1\). Naturally-produced antibiotics and their chemical derivatives have been the backbone of the evolution in therapeutics and infectious disease control in people. Early successes in treating deadly infectious diseases led to wide-spread use of these drugs but at the same time pathogens, craving for survival, also developed strategies to protect themselves from these new weapons by developing resistance\(^2\). From the discovery of the antibiotic streptomycin from *Streptomyces griseus* by Selman Waksman in 1942, there has been a race to screen a wide range of bacteria, especially from the *Streptomyces* genus, for novel bioactive molecules\(^1\). The period 1945-1960s is termed the “golden era” for the discovery of antibiotics as almost all important classes of antibiotics, such as tetracyclines, aminoglycosides, macrolides, and cephalosporins were discovered at that time. In later years however the pace of discovery of antibiotics declined dramatically as scientists became convinced that the antibiotic potential of this genus was exhausted\(^3\). The focus then shifted to chemical modification of the pre-existing drugs to achieve novelty and improved potency\(^4\). New antibiotic discovery from natural sources, although a rewarding commercial pursuit, is cost-prohibitive and challenging in terms of de-replication,
specifically the re-discovery of known molecules\textsuperscript{5}. Despite efforts during the past 40 years, only two new chemical classes of antibiotics, linezolid (anoxazolidinone) in 2000\textsuperscript{6} and daptomycin (acycllic lipopeptide) in 2003\textsuperscript{7} were approved for clinical use. The rapid emergence of multi-drug-resistant bacteria and the paucity of new efficacious antibiotics are major reasons of scare and concern to public health\textsuperscript{8}. Undisputedly, there is a need for concerted efforts to discover novel antibiotics\textsuperscript{9} by employing modern techniques and tools available at our command.

1.2 Streptomyces: Brief History and Life Cycle

Actinomycetes, the ubiquitous and abundant soil organisms that give the characteristic earthy smell to soil, are Gram positive bacteria rich in G+C content. This family is multicellular, exhibits a mycelial habitat and include \textit{Streptomyces}, \textit{Micromonospora}, \textit{Mycobacterium}, \textit{Nocardia}, \textit{Salinospora}, \textit{Saccharopolyspora}. Most dominant and well-studied amongst actinomycetes are the \textit{Streptomyces}\textsuperscript{10}. They reproduce by forming spores and hence were originally classified as fungi. These are best known for their ability to synthesize secondary metabolites, many of which act as antibiotics\textsuperscript{1}. Secondary metabolites are not essential for growth of the organisms but provide diverse survival functions in the hostile environment\textsuperscript{11}. The model organism of this genus \textit{Streptomyces coelicolor} has been studied for both its complex morphological development and its secondary metabolite production\textsuperscript{12}. Figure 1 represents schematics of the life-cycle of \textit{Streptomyces}. Most of the streptomycetes in the soil exist as spores that are semi-dormant and can survive for long periods in low nutrient and water conditions. The life cycle begins with a spore\textsuperscript{10}, which post-germination transforms into one or two
germ tubes that grow into a network of filaments called the substrate mycelium. Substrate mycelium does not undergo cell division but give rise to multi-genomic compartments. As the nutrients become exhausted, the substrate mycelium differentiates into an aerial mycelium at the expense of biomolecules released from the breakdown of the substrate mycelium\textsuperscript{10}. Aerial mycelium erupts upward, away from the substrate mycelium and undergoes cell division to form uni-genomic reproductive spores. Antibiotics are produced as secondary metabolites during the transition from substrate mycelium to aerial hyphae growth probably to defend the food source from neighbouring competitive microorganisms in the soil\textsuperscript{10}. This proves a strong but complex link between stress, growth, morphogenesis and secondary metabolite production. Although non-essential to bacterial survival, these secondary metabolites have found important applications in human medicine as antibacterial, antitumor, antifungal and anti-parasitic drugs\textsuperscript{13}. The contribution of \textit{Streptomyces} to furthering human health can be noted from the fact that greater than 50\% of clinically useful antibiotics find their origin from these bacteria\textsuperscript{10}.

1.3 \textit{Streptomyces} as source of novel antibiotics

\textit{Streptomyces} live in the soil as non-motile bacteria and hence produce an array of secondary metabolites to aid access to scarce nutrients in the soil and to overcome competition with cohort soil microorganisms\textsuperscript{14}. \textit{S. coelicolor} is the most commonly used organism in research as it is easy to culture in the laboratory and the colonies can be easily visualized due to the production of blue and red pigmented antibiotics actinorhodin (ACT)\textsuperscript{15} and undecylprodigiosine (RED)\textsuperscript{16}, respectively. The presence of coloured colonies on culture provides an obvious advantage to study growth characteristics and
Figure 1 - Life cycle of *Streptomyces*. In a nutrient rich condition, the spore germinates to give rise to germ tubes which grows to form multi-genomic substrate hyphae. As the nutrients are depleted the substrate mycelium differentiate to form aerial mycelium that grows upward from the colony to form uni-genomic spores which mature and complete the life cycle.

analyze phenotypes\(^\text{13}\). In spite of protracted efforts, only five antibiotics, the chromosomally encoded ACT, RED, calcium dependent antibiotics (CDA)\(^\text{17}\) and coelimycin P1\(^\text{18}\); and the plasmid encoded methylenomycin\(^\text{19}\) have been characterized from *S. coelicolor* so far. The paradigm however changed a decade ago when the genome sequence of *S. coelicolor* was first published\(^\text{20}\). The availability of powerful DNA recombinant technologies and the total genome sequence data available for *S. coelicolor* have provided inspiration for intense research on the genetic and molecular basis of antibiotic production\(^\text{20}\). With the help of data from genomic analysis it became evident
that *S. coelicolor* is bestowed with an extensive genetic architecture capable of producing an average of 20-30 additional undetected secondary metabolites\(^\text{14}\). Interestingly, the set of genes responsible for production of individual antibiotic or secondary metabolite were found to be clustered on the chromosome\(^\text{21}\). Since the products transcribed by these gene clusters are not detected on culture due to extremely low level of expression, these are now termed ‘silent’ or ‘cryptic’ gene clusters\(^\text{22}\). Subsequent studies in other *Streptomyces* proved that this phenomenon is wide-spread in the genus and possibly in other organisms, although the secondary metabolites produced are often species-specific. In *S. coelicolor* alone, 31 secondary metabolite gene clusters (29 from the chromosome and two from the linear plasmid SCP1) have been predicted so far, of which the products of 14 are cryptic, whereas in *Streptomyces avermitilis*, a source of anti-parasitic compound avermectin, at least 37 secondary metabolites gene clusters have been predicted, out of which 27 metabolites are cryptic\(^\text{23}\).

The organization of the gene clusters responsible for synthesis of individual antibiotics is also very intriguing. The clusters consist of genes responsible for core biosynthesis, transcription regulation, tailoring, self-resistance and transport, arranged in close proximity\(^\text{24-26}\). It is reasonable to assume that for timely expression and repression of such a vast array of around 819 secreted proteins in *S. coelicolor* involved in nutrient scavenging and numerous more responsible for secondary metabolite production, there must be precise mechanisms of genetic and metabolic regulation. The emphasis on regulation is reiterated from the observation that around 12.3% of the proteins in this genus are predicted to have regulatory functions\(^\text{20}\). In *S. coelicolor*, the 31 predicted
secondary metabolite gene clusters include type I modular, type II modular and iterative polyketide synthases (PKSs), chalcone synthases, non-ribosomal peptide synthetases (NRPSs), and terpene cyclases\textsuperscript{20,23}. Understanding the underlying processes, mechanisms and regulatory pathways will provide vital clues to devise strategies to unravel new antibiotics and improve yields of known antibiotics to render their commercialization possible.

1.4 Regulation of secondary metabolite production

A general scheme of the known regulatory pathways of a number of antibiotics synthesized by \textit{S. coelicolor} is depicted in Figure 2. It is now established that the production of individual secondary metabolites is the net outcome of the recognition of a specific stimulus, the transduction of this signal to produce specific regulators, the activation of the biosynthetic gene cluster by these regulators to produce the secondary metabolite and finally the transport of this molecule\textsuperscript{27,28}. Although the need for understanding secondary metabolite regulation was felt as early as 1970, only recently has it received intense attention and success due to the availability of powerful biotechnology tools. As a general rule, when \textit{Streptomyces} (and possibly other antibiotic-producing bacteria) are cultured in environments that cause nutritional stress, the stationary growth phase coincides with onset of secondary metabolite production\textsuperscript{21}. Taking advantage of this observation, the industry has devised strategies to maximise antibiotic production by manipulating media and fermentation conditions\textsuperscript{29}. Nutrient factors, such as phosphate\textsuperscript{30}, trace elements (Fe, Zn, Mn)\textsuperscript{31}, nitrogen and carbon sources\textsuperscript{32} and metabolites like N-acetylglucosamine (GlcNAc)\textsuperscript{33} have been shown to influence
antibiotic production in streptomycetes. These nutrients exert their effects directly and indirectly by activating or repressing transcription factors and regulatory proteins. For example, sustained nutrient deprivation in *S. coelicolor* activates the stringent response to augment the highly phosphorylated guanosine nucleotide (ppGpp) pool, which affects levels of transcription factors *redD* and *actII-ORF4* and thus impacts the production of respective antibiotics RED and ACT\(^{21}\). Similarly, the presence of inorganic phosphate exerts negative control over antibiotic production which is mediated through the two component system (TCS) PhoP/R\(^{30}\).

Recent studies on the genetic basis of antibiotic synthesis regulation have unravelled intriguing biological mysteries\(^{34,35}\). As a general scheme, control is exerted through regulatory proteins transcribed by the genes that are usually present within the corresponding antibiotic gene cluster\(^{36,37}\). Considering the spectrum of their effects, these encoded regulatory proteins can be grouped into two distinct classes: (a) pleiotropic regulators that affect both antibiotic production and morphogenesis or multiple antibiotic biosynthesis pathways without affecting morphogenesis and (b) pathway-specific regulators, which regulate only one antibiotic gene cluster\(^{21,24}\). In *S. coelicolor*, there are over 28 pleiotropic regulators and about 13 pathway-specific regulators\(^{37}\). Pathway-specific regulators usually belong to the SARP (Streptomyces Antibiotic Regulatory Protein) family that preferentially act as transcriptional activators\(^{21,38}\). In *S. coelicolor*, *actII-ORF4, redD* and *cdaR* encode SARP for production of ACT, RED and CDA, respectively\(^{39,40}\). In the case of antibiotics regulated through the pathway-specific process, the biosynthetic gene cluster is governed by a single regulatory
Figure 2 - Complex regulation of antibiotic production in *S. coelicolor* by different signaling pathways. AfsK/R is the serine/threonine kinase pathway where AfsK autophosphorylates on receiving a signal and transfers the phosphoryl group to AfsR and its activity is modulated by KbpA (AfsK binding protein A). The activated AfsR stimulates the transcription of *afsS*. *afsS* transcription is also stimulated by PhoP, which is activated by PhoPR during phosphate starvation. AfsS has been shown to be involved in stimulation of antibiotic synthesis. AbsA1/A2 and AfsQ1/Q2 are TCS. On receiving an unknown signal (shown as ‘?’) AbsA1 phosphorylates AbsA2 which plays a role in inhibition of antibiotic synthesis. On the other hand, AfsQ2 on receiving an unknown signal (shown as ‘?’) phosphorylates AfsQ1 which stimulates antibiotic production. SigQ protein may be involved in antagonizing the function of AfsQ1/Q2. A microbial hormone, A factor relieves the repression on the regulatory protein AdpA which in turn stimulates *strR*, the pathway-specific regulator of streptomycin. AtrA has been shown to stimulate both antibiotic production and the expression of GlcNac transporter whereas, DasR negatively regulates antibiotic production. GlcNac has been shown to stimulate antibiotic production in *S. coelicolor*. As it is internalized by the cell it gets converted to glucosamine-6-P (GlcN-6-P) by PTS and binds to DasR inhibiting its antibiotic inhibition effect, thus stimulating antibiotic production.
gene which exerts its control in tandem with multiple signal inputs generated by diverse cellular processes\textsuperscript{36}. For example, in \textit{S. coelicolor}, production of ACT is regulated by a single gene producing the regulatory protein ActII-ORF4, which in turn controls five transcription units of the ACT gene cluster and is directly controlled by at least eight known regulatory proteins including global regulators\textsuperscript{41}.

In contrast, pleiotropic regulators have effects on multiple metabolic pathways, influencing physiological responses, morphogenesis and antibiotic production. The list of pleiotropic regulators is quite diverse and includes, signaling molecules (such as ScbA and A factor)\textsuperscript{42,43}, TetR family regulators (such as AtrA)\textsuperscript{44,45}, cyclic AMP receptor protein (crp)\textsuperscript{46}, and TCSs (such as AbsA1/2, AfsK/R/S, PhoP/R and AfsQ1/2)\textsuperscript{47-50}. These regulatory proteins mediate their effects through intricate but well-coordinated processes, often converging at the regulators specific to a single metabolic pathway. An interesting example is the involvement of a pleiotropic regulator AtrA on streptomycin production in \textit{S. griseus}, which activates \textit{strR}, the pathway-specific regulator of streptomycin by binding to its promoter region\textsuperscript{45}. Interestingly, \textit{strR} is the target for another regulatory protein AdpA which, in turn, is the sole target for a repressor protein ArpA (A-factor receptor protein) (Figure 2). In the native, inactive state, ArpA remains bound to \textit{adpA}, but when the cellular levels of gamma-butyrolactone (commonly called A-factor) reach a specific threshold, ArpA is released from the \textit{adpA} promoter region, which in turn is regulated in a complex way by its own gene product and several other pleiotropic regulators\textsuperscript{43,51,52}. AtrA also regulates the transport of glucosamine-6-phosphate (GlcN-6-P). It is an allosteric effector molecule of another key regulator of antibiotic biosynthesis,
DasR, a GntR family member\textsuperscript{53,54} (Figure 2). GlcN-6-P is formed by phosphorylation of N-acetylglucosamine (GlcNAc), which is released during the lytic dismantling of substrate mycelium under nutrient stress conditions\textsuperscript{55,56}. GlcNAc has been shown to stimulate antibiotic production in \textit{S. coelicolor}, \textit{S. clavuligerus}, \textit{S. griseus}, \textit{S. hygroscopicus} and \textit{S. venezuelae}\textsuperscript{33}. Binding of GlcN-6-P to DasR inhibits its DNA-binding ability, resulting in loss of its transcriptional repression on \textit{actII-ORF4}, \textit{redZ} and GlcN-6-P specific phosphotransferase transport system (PTS)\textsuperscript{54}. These are classic examples of metabolic and genetic connections between morphological differentiation and secondary metabolite production and the influence of pleiotropic regulators on the up and down regulation of the pathway-specific regulators. From above it is evident that the regulatory pathways for secondary metabolite production are inter-related and complex; its manipulation could be a simple and direct way to activate the cryptic gene clusters and harness new antibiotics.

1.5 \textbf{Strategies to improve secondary metabolite production}

A number of strategies have been adopted to improve secondary metabolite production, and a summary is described in Table 1. But since my thesis work is related to regulatory proteins and their manipulation, this aspect is discussed in greater detail. There is an overwhelming expectation that activation of cryptic gene clusters might be a plausible approach to identify undetected antibiotics and other pharmacological agents, thus unleashing the full metabolic potential of organisms like \textit{Streptomyces}. To achieve this goal, it is necessary to understand the physiological signals, regulatory mechanisms and biosynthetic pathways of target molecules. In order to activate cryptic gene clusters,
Table 1. Summary of strategies to improve secondary metabolite production

<table>
<thead>
<tr>
<th>Approach</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conventional approach</strong></td>
<td></td>
</tr>
<tr>
<td>Manipulation of nutrient conditions</td>
<td>Production of a macrolide antibiotic spiramycin in <em>S. ambobaciens</em> is inhibited by addition of glucose, 2-deoxyglucose and inorganic phosphate. Production of daptomycin in <em>S. roseosporus</em> requires addition of decanoic acid.</td>
</tr>
<tr>
<td>Manipulation of stress response</td>
<td>In <em>S. venezuela</em>, ethanol shock or growth at high temperature (42 °C) have been shown to efficiently produce jadomycin B.</td>
</tr>
<tr>
<td>Random mutagenesis</td>
<td>Clavulanic acid and Rapamycin yields were improved in <em>S. clavuligerus</em> and <em>S. hygroscopicus</em> by random mutagenesis.</td>
</tr>
<tr>
<td><strong>Genome mining approach</strong></td>
<td></td>
</tr>
<tr>
<td>Identification of cryptic product(s) by prediction of physiochemical properties</td>
<td>In the marine actinomycete <em>Salinospora tropica</em>, sequence analysis of a novel modular PKS suggested its product to be a lysine primed polylene macrolactam. Since polylene absorbs UV light and this physiochemical property guided the isolation of a series of polylene macrolactams, one of which was salinilactam A.</td>
</tr>
<tr>
<td>The Genomes isotopic approach</td>
<td>Combination of genome sequence analysis, and substrate prediction and isotope-guided product isolation to identify novel compounds. For example, In <em>Psuedomonas fluorescens</em> Pf-5, feeding 15N labeled leucine and identifying molecules containing 15N leucine led to discovery of a novel bioactive cyclic lipopeptide, orfamide A.</td>
</tr>
<tr>
<td>Gene knockout/ comparative metabolic profiling</td>
<td>In <em>S. coelicolor</em>, inactivation of NRPS encoding <em>cchH</em> gene and comparative metabolic profiling led to identification of a ferric-iron-chelating tripeptide called coelichelin.</td>
</tr>
<tr>
<td>Heterologous gene expression of biosynthetic gene cluster and comparative metabolic profiling</td>
<td>Heterologous expression of a putative operon of three genes within <em>S. coelicolor</em> A(3)2 methylenomycin biosynthetic gene cluster in <em>S. coelicolor</em> M512, lacking SCP1 plasmid, led to identification of methylenomycin furans, an inducers for methylenomycin antibiotics production.</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
</tr>
<tr>
<td>Use of small molecule probes</td>
<td>Improvement in secondary metabolite production can be achieved by adding synthetic small molecule probes. For example, addition of ARC2 has led to increased production of desferrioxamine B/E in <em>S. pristinaespiralis</em>, doxorubicin and baumycin in <em>S. peucetius</em> and an unknown metabolite in <em>Streptomyces kutzneria</em>.</td>
</tr>
<tr>
<td>Ribosome engineering</td>
<td>Resistance to known antibiotics by targeting ribosomal proteins S12 or RNA polymerase RNAP or translation factors enhances yields of cryptic secondary metabolites. Mutation in S12 and or RNAP in 6% non-<em>Streptomyces</em> actinomycetes species and 43% of the <em>Streptomyces</em> species led to production of an unknown class of antibiotics named piperidamycin.</td>
</tr>
</tbody>
</table>
diverse strategies are available, ranging from simple nutrient manipulation\textsuperscript{57}, and random mutagenesis\textsuperscript{58,59} to genetic engineering\textsuperscript{60,61}. Thanks to recent advances in molecular genetics and bioinformatics technologies, a better understanding of the secondary metabolite regulation and an open access to sequenced genome databases of antibiotic-producing organisms, the metabolic engineering on the regulatory elements have received widespread acceptance\textsuperscript{62}. There are two distinct possibilities in this approach. First, the pathway-specific regulators can be manipulated in native host to achieve activation or repression. In the second approach, pleiotropic regulators can be manipulated in native or heterologous hosts\textsuperscript{62}.

1.5.1 Manipulation of pathway-specific regulators: Pathway-specific regulator engineering is usually directed at improving the yield of secondary metabolites in native or heterologous hosts. For instance, it was possible to improve the yield of fredericamycin by 5.6-fold by over-expression of the pathway-specific activator \textit{fdmR1} in native host \textit{S. griseus}\textsuperscript{71}. In some cases where the cryptic gene cluster can be predicted from genome mining, it is possible to adopt the pathway-specific manipulation approach. A successful example of this approach is reported in\textsuperscript{22}. Genomic analysis of \textit{S. ambofaciens} predicted a type I modular PKS composed of 25 genes and spanning 150 kb, of which the product was unknown since it was not produced under laboratory growth conditions. Constitutive expression of the regulator \textit{samR0467} which is located within this PKS gene cluster triggered the production of four 51-membered glycosylated macrolides named stambomycins A-D, as the metabolic product of the gene cluster\textsuperscript{22}. It is also possible to stimulate antibiotic production in native hosts by eliminating the
influence of a negative pathway specific regulator. For example, it is established that production of platencinamycin and platencin in *S. platensis* MA7327 is encoded by a single gene cluster\textsuperscript{72,73}. Inactivation of a negative regulatory gene *ptmR1* in the native host led to over-expression of these two antibiotics by 100-fold compared to the wild strain\textsuperscript{74}. Although fairly simple and straight-forward, the major constraint with the technique based on engineering of the pathway-specific regulator is that it leads to regulation of a single secondary metabolite. This approach is of high-risk as the success rate could be low.

**1.5.2 Manipulation of pleiotropic regulators in native hosts:** The unlocking of cryptic gene clusters is also possible by genetically engineering pleiotropic regulators. This can be done in the native host or the engineered regulator can be expressed in heterologous hosts. This approach, although relatively complicated, has obvious advantages over the manipulation of pathway-specific regulators, as multiple secondary metabolite gene clusters can be activated, leading to induction of several novel molecules or enhanced production of known molecules. A successful example is the manipulation of the pleiotropic regulator PhoP. In *S. coelicolor*, this protein has been shown to regulate many genes involved in antibiotic production and morphological differentiation such as *afsS, atrA, bldA, bldC, bldD, bldK, bldM, cdaR* and *scbR-scbA*\textsuperscript{75}. PhoP belongs to a TCS called PhoP/R. In *S. lividans*, deletion of PhoP/R has been shown to increase production of ACT and RED by 5-fold and 12-fold, respectively\textsuperscript{49}. Expression of engineered pleiotropic regulators in heterologous hosts also offers potential for discovering novel molecules from cryptic clusters. Another advantage is that secondary metabolite production can be
altered in heterologous hosts without any prior knowledge of the host strains or the antibiotic regulatory system\textsuperscript{76}. Because these regulatory pathways are widely conserved in streptomycetes a regulator from one species can be used in other species\textsuperscript{46,77}. In \textit{S. coelicolor}, over 28 pleiotropic regulators have been identified so far\textsuperscript{69}. A number of these proteins, such as, AfsK/R/S, AtrA, DasR, PhoP/R, AfsQ1/Q2 are well characterized and their heterologous expression in diverse hosts have led to either the yield improvement of antibiotics or the activation of cryptic gene clusters\textsuperscript{33,44,75,78,79}.

1.5.3 Heterologous expression of pleiotropic regulators to activate cryptic gene cluster: Currently, it is well accepted that the majority of pleiotropic regulators of secondary metabolite production belong to the TCS family and are abundant in \textit{S. coelicolor} and other actinomycetes. Genomic analysis predicts that \textit{S. coelicolor} has the potential to encode 67 TCSs, 84 sensor kinases (SK) and 80 response regulators (RR), suggesting that it is capable of responding to a wide range of environmental signals\textsuperscript{28}. In TCSs, consequence to extracellular stimuli the first component, the SK, an integral membrane protein, phosphorylates the second cognate RR component leading to its binding to specific promoter regions and activation or repression of the target genes\textsuperscript{27,80}. Engineering of TCSs family of pleiotropic regulators offers practical and exciting opportunities to either unlock cryptic gene clusters or over-express the genes responsible for the production of known antibiotics to achieve better yield. This genetic manipulation is possible both at the SK level and at the RR component. An important breakthrough employing this technique has been reported from our laboratory\textsuperscript{77}. In \textit{S. coelicolor}, AbsA1/2 is a TCS that regulates multiple antibiotic syntheses (Figure 2). It has been
reported earlier that the absence of phosphorylated AbsA2 led to early and hyper-production of antibiotics, indicating its role in negative regulation of ACT, RED and CDA in *S. coelicolor*. AbsA1/A2 has shown to be the global negative regulator of the antibiotic production. A mutated allele of AbsA1, AbsA1<sub>H202A</sub>, which is deficient in its kinase activity, has been used to activate the cryptic gene cluster<sup>77</sup>. The mutant AbsA1<sub>H202A</sub> was introduced into six environmental soil isolates and nine characterized antibiotics producing strains. The most striking result was seen in one strain *S. flavopersicus*, wherein enhanced pulvomycin synthesis killed *B. cenocepacia*, an opportunistic pathogen of cystic fibrosis patients. Though the molecule pulvomyin is not novel, it was fascinating because pulvomyin is not known to be produced by the nascent *S. flavopersicus*. This provides convincing evidence that heterologous expression of pleiotropic regulators could lead to activation of cryptic gene clusters<sup>77</sup>. It is notable that AbsA1-like operon has found to be conserved in diverse strains of *Streptomyces* such as *S. cinnamycin; S. griseochromogenes, S. cinnamoneus, S. fungicidins*, and *S. fradiae*<sup>81-83</sup>.

In *S. coelicolor*, AfsR, a SARP family regulatory protein, is a RR of a serine/threonine kinase system AfsK/R/S<sup>84</sup>. To become active, it must be phosphorylated by its cognate SK AfsK, which is modulated by KbpA (AfsK binding protein A)<sup>85,86</sup>. The trigger for activation of this system is S-adenosyl-L-methionine (SAM)<sup>87,88</sup> (Figure 2). In addition to AfsK, two other kinases, PkaG and AfsL are also capable of phosphorylating AfsR<sup>84</sup>. Phosphorylation of AfsR greatly enhances its DNA binding activity and the cascade culminates in antibiotic production indirectly by activating a 63 amino acid long sigma-like protein AfsS<sup>84</sup>. The homologue of *afsR* and *afsS* exists in other *Streptomyces* strains,
such as afsR-p in S. peucetius and ssmA in S. noursei, respectively. As such, manipulation of both afsS and afsR offers potential to activate cryptic genes in heterologous hosts and produce novel antibiotics. Over-expression of afsR-p from S. peucetius into a heterologous host S. venezuelae ATCC 15439 has led to the increased production of pikromycin by 2.6-fold\textsuperscript{89}. Similarly, over expression of afsR-p in S. lividans, S. clavuligerus and S. griseus has been shown to increase the production of ACT (2.6-fold), clavulanic acid (1.5-fold) and streptomycin (slight increase), respectively\textsuperscript{78}. In S. coelicolor, S. lividans and S. griseus, over expression of afsS has been shown to enhance antibiotic production\textsuperscript{90-92}. Expression of afsS is also controlled by PhoP/R, a TCS that controls the phosphate uptake in phosphate limiting conditions (Figure 2). This indicates competition between AfsR and PhoP for regulating afsS expression due to overlap of the binding site in the promoter region\textsuperscript{93}. Thus, manipulation of PhoP can indirectly influence antibiotic production.

Another example of TCS that offers potential for manipulation and over-expression of cryptic gene clusters is the AfsQ1/AfsQ2/SigQ. Figure 3 depicts the schematic representation of the AfsQ1/Q2/SigQ gene organisation. AfsQ1/2 was identified in S. lividans for its ability to stimulate ACT, RED, and CDA production when introduced in a high copy number plasmid\textsuperscript{94}. In AfsQ1/2, AfsQ1 is the RR and AfsQ2 is the HK, which in turn acts as a sole phosphate donor for AfsQ1\textsuperscript{50} (Figure 4). The third gene in the afsQ operon, afsQ3 encodes a putative lipoprotein that may be an accessory protein to the AfsQ TCS\textsuperscript{95} (Figure 3). Several studies have indicated that disruption of either afsQ1 or afsQ2 in S. coelicolor resulted in decreased ACT, RED, CDA, and a
yellow pigment, named yCPK, and rapid growth of aerial mycelium when grown on a defined minimal medium (MM) containing high concentration of glutamate as the sole nitrogen source\textsuperscript{79}. The results showed that AfsQ1/Q2 stimulated ACT, RED, and CDA production directly through the pathway-specific activator genes \textit{actII-ORF4}, \textit{redD}, and \textit{cdaR}, respectively\textsuperscript{79} (Figure 4). This indicates that AfsQ1 has pleiotropic effects on

\textbf{Figure 3} - Schematic representation of organization of \textit{afsQ-sigQ} genes: \textit{afsQ} and \textit{sigQ} are divergent genes. \textit{afsQ1}, \textit{afsQ2}, \textit{afsQ3} encode for a sensor kinase, histidine kinase and a putative lipoprotein respectively. \textit{sigQ} which is transcribed in the opposite direction encodes for a putative sigma factor.

\textbf{Figure 4} - Schematic representation of AfsQ two component signal transduction system: AfsQ2 responds to unknown cellular or environmental signal by phosphorylating AfsQ1. AfsQ1-P then activates antibiotic synthesis through pathway specific activator genes.
antibiotic production and morphological differentiation. It was also shown that the AfsQ1/Q2 system regulates expression of the divergently located $\text{sigQ}$, encoding a putative sigma factor. SigQ might be involved in antagonizing the function of AfsQ1/2 in $S. \text{coelicolor}$, in a media-dependent manner as its deletion led to precocious hyper-production of ACT, RED, CDA and delayed sporulation in the same glutamate-based MM$^{50,79}$.

AfsQ1/2 is also involved in maintaining the metabolic homeostasis and negatively regulates genes involved in nitrogen assimilation by competing with the central regulator of nitrogen metabolism, GlnR$^{79}$. In addition to nitrogen metabolism, AfsQ1 also seems to be involved in regulation of phosphate and carbon metabolism, indicating that the protein enables the microbes to rapidly adapt to the complex surrounding environment$^{79}$. Interestingly, multiple amino acid sequence alignment in seven streptomycetes strains reveals that AfsQ1 is widely conserved implying that the role of AfsQ1/Q2 system is widespread (Figure 5). Evidence of AfsQ1 involvement in regulation of primary metabolism, secondary metabolism and morphogenesis makes it one of the important global regulators that can be further manipulated in different $S. \text{streptomyces}$ strains for antimicrobial production.
Multiple amino acid sequence alignment of AfsQ1 from *Streptomyces* species. AfsQ1 protein sequence from *S. coelicolor* and homologous proteins from *S. scabiei*; *S. albus* J107; *S. avermilitis* MA-4680; *S. coelicolor*; *S. clavuligerus* ATCC 27064; *S. griseus*; and *S. venezuelae* ATCC 10712 were obtained using StrepDB, NCBI and Broad institute databases. The alignment was generated using clustalw2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The aspartate (D) residue marked in red is the site of the phosphorylation.

1.6 Project Rationale

Nancy McKenzie’s work in our laboratory on the activation of cryptic gene clusters by heterologous expression of AbsA1H202A laid the foundation for use of this technique. Subsequently, the same technique was employed by graduate students in our laboratory Michael Hart and Kyle Salci who over-expressed the pleiotropic regulator *afsQ1* in the wild isolate WAC04657 and saw increased antimicrobial activity against indicator strains such as *S. aureus*, *B. subtilis* and *M. luteus*. Since the signal required by this regulatory system is unknown, a constitutively active mutant allele *afsQ1D52E* (*afsQ1**) was synthesized de novo* by GenScript. The phosphorylation site of AfsQ1 is the aspartate residue at the 52\textsuperscript{nd} position (Figure 5). This residue was replaced by a glutamate to mimic the functional phosphorylation state and results in *afsQ1* remaining in a
constitutively active state\textsuperscript{96}. Another team of graduate students, Tomas Gverzdys and Daniel Socko, over-expressed the pleiotropic regulator $afsQ1^*$ in WAC04657, and found that the engineered strain significantly increased antimicrobial activity in colony diffusion assays. Specifically, activity against multi-drug resistant Gram-negative bacteria such as *Acinetobacter baumannii* B0098426R, *Pseudomonas aeruginosa* PA01 and *Klebsiella pneumoniae* was seen only in WAC04657 expressing $afsQ1^*$. Coupled with early LC-MS data, the evidence suggests the activation of a cryptic gene cluster (Personal communication, Gverzdys T, Nodwell J). The ability of this regulator to modify the antibiotic profile of WAC04657 and the existing literature implicating its involvement in the regulation of multiple antibiotics and its control over primary metabolite pathways makes $afsQ1^*$ a promising candidate to further exploit its capability in diverse *Streptomyces* strains.

For my thesis research, I expressed $afsQ1^*$ in a library of wild-isolate actinomycetes from the lab of Dr. Gerry Wright and looked for expression of secondary metabolites, in particular antibiotics, by performing standard growth inhibition assays. The putative novel molecules were then purified and partially-characterized by using analytical techniques such as High Performance Liquid Chromatography (HPLC), Liquid Chromatography-Mass Spectrometry (LC-MS).
Chapter 2: Materials and Methods

2.1 Bacterial strains, vectors and growth conditions

*E. coli* strain ET12567 [pUZ8002] used as the donor in the intergeneric conjugation, is a methylation defective strain \((dam-13::\ Tn9\ dcm-6\ hsdM)^{97,98}\). pUZ8002 is a derivative of RK2, which can transfer other plasmids with *oriT*, but is not self-transmissible due to mutation in its *oriT*\(^{99}\). All the vectors used in the study are mentioned in the Table 2. The *E. coli* donor strains were always grown in liquid Luria-Bertani (LB) media at 37 °C with shaking at 200 rpm. Conjugation of vectors was carried on MS media\(^{10}\). The 120 wild isolates used in this thesis are from Wright Actinomycetes Collection (WAC) and are listed in Table 3. All WAC strains were grown on MYM media at 30°C unless mentioned otherwise (refer to the media recipe section).

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<tr>
<th>Donor Strains or Vectors</th>
<th>Genotype &amp;/ Description</th>
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<tr>
<td>ET12567 [pUZ8002](^{97})</td>
<td>(dam-13::\ Tn9\ dcm-6\ hsdM, chl^r, kan^r)</td>
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<td>Vectors</td>
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<td>pSET152-ermE^±p-null (EV)(^{100})</td>
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<td>pSET152-ermE^±p-afsQ1 (Q1)(^{101})</td>
<td>Wild type <em>afsQ1</em> vector, apr^r</td>
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<tr>
<td>pSET152-ermE^±p-afsQ1(_{D52E}) (Q1*)(^{101})</td>
<td>Mutant allele <em>afsQ1</em> vector, apr^r</td>
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Table 2: List of strains and vectors used during conjugation
## Table 3: List of wild isolate strains from the Wright Actinomycetes Collection (WAC) library

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### 2.2 Conjugation and screening

#### 2.2.1 Medium-throughput conjugation

Conjugation was carried out using 96-well plates containing 200 µl of MS agar in each well. Wild actinomycetes colonies used for conjugation were arrayed on a 96-well plates containing MYM media. The donor *E. coli* ET12567/pUZ8002 containing EV, Q1 and Q1* was inoculated in LB broth with 50 µg/ml apramycin, 25 µg/ml chloramphenicol, and 50 µg/ml kanamycin and grown overnight to an OD$_{600}$ of ~0.4. On the following day, the donor cells were washed thrice with LB (without antibiotics) and then resuspended in 1/10$^{th}$ of the original volume of LB used for washing. Ten microlitre of the 10x donor *E. coli* were overlaid in the 96-well MS plates and dried. Once dry, using the sterile cryoreplicator, the sporulated actinomycetes were pinned from the master plate to the 96-well MS plates containing the donor cells and incubated at 30°C for 12-14 hours, without shaking, and face up. Using an automatic fluid distributor (µ-fill) the plates were overlaid with 30 µl of solution containing 0.125 mg/ml nalidixic acid and
0.313 mg/ml of apramycin. Positive controls were actinomycetes colonies with donor cells and were overlaid with only nalidixic acid (25 µl of nalidixic acid in 5 ml of sterile water). Positive controls were carried out to check the growth of the 120 WAC strains. Negative controls were actinomycetes colonies pinned on MS plates without the donor *E. coli* cells to select the apramycin resistant WAC strains. They were overlaid with the same amount of apramycin and nalidixic acid as that for the conjugation plates. The plates were incubated at 30°C until the modified colonies sporulated. It usually take two-weeks before the exconjugants appear. The conjugation was done in triplicate and exconjugants in any two replicates were considered as positive.

2.2.2 PCR confirmation of the ex-conjugants

Genomic DNA of the WAC00263 and WAC00226 were prepared by inoculating 2 µl of the spore stock into 5-10 ml of the MYM media. Few glass beads were added to the media to improve aeration and shearing of the mycelium. The culture was grown for 2 days and the genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue kit. Primers (Table 4) were designed to amplify 565 base pair (bp) region of EV or 1.2 kb region in case of Q1*. PCR was carried out on 50 ng of the genomic DNA using Taq DNA polymerase. The PCR reaction was carried out with the following program: initial denaturation at 95°C for 2 minutes, followed by 30 cycles of amplification (30 seconds of denaturation at 95°C, 30 seconds of annealing at 52°C, and 40 seconds of extension at 72°C) and finally, a final extension of 2 minutes at 72°C.
Table 4: *Oligonucleotides used in this study.

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<th>Primer type</th>
<th>Oligonucleotide sequence</th>
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<td>Forward primer</td>
<td>5’-ACGACGGCCAGTGCCAAGCT-3’</td>
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<tr>
<td>Reverse primer</td>
<td>5’-CGGCTCGTATGTTGTGGAATTGT-3’</td>
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* These were ordered from Mobix Laboratory at McMaster University.

2.2.3 Colony diffusion assays

In order to compare the antimicrobial molecules produced by the strain containing Q1*, EV, as well as wild type (WT), colony diffusion assays were performed on a solid media plate. This assay requires equal concentration of the spores. $10^5$ spores/5 µl were spotted on the same solid media plate and allowed to dry. The plates were then incubated at 30°C for 7 days and then were overlaid with the indicator organism. Overnight culture of the indicator organism was diluted to OD$_{600}$ of 0.1 by adding it to the molten soft-LB agar. Five millilitre of the molten soft-agar was overlaid on top of the plate and allowed to solidify. The plate was incubated overnight at 30°C. The antimicrobial activity was indicated by measuring the zone of inhibition around the colonies.

2.3 Metabolite Profiling, Purification and Characterization of the Molecules

2.3.1 Preparation of crude extracts for comparative metabolite profiling

The spores of the strain ($10^5$ spores/5 µl) were inoculated on a 60 mm solid media(s) plate, containing either MYM or R2YE$^{10}$, and incubated for specific number of days (depending on the number of days at which antimicrobial activity was seen in the colony diffusion assay), following which these were extracted overnight with butanol. These experiments were done in duplicates and included media controls. Following
extraction, the solvent was filtered using Whatman Grade 1 filter paper and evaporated using Genevac. The pellet was then re-suspended in 1:1 MeCN:H₂O.

2.3.2 Preparation of large scale crude extracts for comparative metabolite profiling

Five microlitre of diluted spore stock (200 µl in 800 µl of saline) of the strain was inoculated on 20 solid media plates. After seven days of incubation, the agar was chopped and extracted overnight with ~1L of butanol. On the following day, the solvent was evaporated using Genevac and the pellet was re-suspended in 8-9 ml 1:1 MeCN: H₂O.

2.3.3 LC-MS specifications

LC-MS analysis was performed on an Agilent 1200 ‘‘RR’’ series LC system coupled to a Bruker microTOF II with an ESI ionization source. LC was carried out at 40°C using a Phenomenex Kinetex C18 column (50 × 2.1 mm, 2.6 µm, 100 Å). The solvents used were, (A) Water containing 0.1% formic acid and (B) MeCN containing 0.1% formic acid. The gradient flow used was: flow of 0.2 ml/min, 0.5 min 5% B, 5–9 min 95% B and 10–15 min 5% B. The MS conditions were set to a capillary voltage of 4.5 kV for positive mode; nebulizing gas pressure (N₂) of 3 bar, dry gas flow rate (N₂) of six l/min, temperature at 200°C, and a scan rate of 1 Hz.

2.3.4 Purification of the molecules from WAC00263 by HPLC

Molecule 1082 [M+2H]²⁺

Gradient elution was used to purify the molecule 1082 [M+2H]²⁺. Elution was performed on an XSelect column equipped with a reverse-phase C18 (10 mm ×150 mm) 5 µm, 100 Å’. The sample 1082 [M+2H]²⁺ was separated using an isocratic elution of
50% acetonitrile (0.1% [vol/vol] formic acid) to 50% water (0.1% [vol/vol] formic acid) at 35°C over 5 min, with a flow rate of 5 ml/min. The compound 1082 [M+2H]^{2+} was collected at the retention time of 3.4 minutes. The purified compound 1082 [M+2H]^{2+} was evaporated to dryness by centrifugal evaporation (Genevac) and lyophilized (Labconco FreeZone). In order to check the purity, the lyophilized sample was re-injected into the HPLC. The molecule was found to be soluble in dimethylsulfoxide (DMSO) and methanol.

**Molecule 782 [M+H]^+**

Purification was performed on a column equipped with a reverse-phase C18 (10 mm ×150 mm) 5 µm, 100 Å. The crude extract of R2YE was pre-fractionated on a Strata X specs 33 µ polymeric reverse phase column using different solvent composition to determine the concentration of the solvent at which the molecule was eluted. Ten millilitre of each of the following solvent compositions were used: (a) 100% water (b) 25% acetonitrile (MeCN) + 75% water (c) 50% MeCN + 50% water (d) 75% MeCN +25% water (e) 100% MeCN.

The molecule 782 [M+H]^+ was separated using the gradient of 50% to 90% acetonitrile (0.1% [vol/vol] formic acid) at 35°C over 8 min, with a flow rate of 1 ml/min. The compound 782 was eluted at 6.1 minutes. The purified compound 782 [M+H]^+ was evaporated to dryness by centrifugal evaporation (Genevac) and lyophilized (Labconco FreeZone). In order to check the purity, the lyophilized sample was re-injected into the HPLC. The molecule was found to be soluble in DMSO and methanol.
2.4 Antimicrobial Activity Testing

2.4.1 Disk diffusion assay

a) Comparative assay for butanol extracts

Eighty microlitre of the crude extract was added to a 6 mm filter disk. Twenty microlitre of the extract was added first and allowed to dry before adding the next 20 µl until the final target volume was achieved. The assays were performed on the LB solid media plate. The indicator strains were inoculated in the LB broth and grown overnight at 37°C. The overnight cultures of indicator organisms such as *E. coli*, *B. subtilis* and *M. luteus* were diluted to OD600 of 0.1. One ml of the diluted culture was added to the petri-plate and allowed to dry. Disks containing the extracts, as well as media and solvent control disks, were placed on the LB plates containing the indicator organisms. The plates were incubated at 37°C overnight. Antimicrobial activity in the extracts was indicated by the zone of inhibition around the disk.

b) Assay with pure compound

The stock solution (10 mg/ml) of pure compounds 1082 [M+2H]2+ and 782 [M+H]+ were prepared by dissolving it in DMSO. Fifty µg/disk of the pure compound was added to the 6 mm diameter disk; same amount (20 µl) of DMSO was added to the second disk (solvent control) and dried. Disk diffusion assay against bacteria (Table 5) was performed as described previously. Fungi *C. neoformans* H99 virulent and *S. cerevisiae* (Table 5), were inoculated in YPD broth and grown overnight at 30°C. On the following day, the overnight culture was diluted to OD530 of 0.1. One ml of the diluted culture was added to the petri-plate and allowed to dry. The disks were placed on the
dried YPD plates and incubated overnight 30°C. Antimicrobial activity in the extracts was indicated by the zone of inhibition around the disk.

**Table 5: List of indicator strains against which pure compound was tested**

<table>
<thead>
<tr>
<th>Laboratory strains</th>
<th>Indicator Strains</th>
<th>Pure Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli ATCC# 25922</td>
<td>1082, 782</td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis #168</td>
<td>1082, 782</td>
<td></td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>1082, 782</td>
<td></td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>1082, 782</td>
<td></td>
</tr>
<tr>
<td>Methicillin Resistant <em>Staphylococcus aureus</em> (MRSA)</td>
<td>1082, 782</td>
<td></td>
</tr>
<tr>
<td>Vancomycin Resistant <em>Enterococcus</em> (VRE)</td>
<td>1082, 782</td>
<td></td>
</tr>
<tr>
<td>Non-resistant <em>Staphylococcus aureus</em></td>
<td>1082, 782</td>
<td></td>
</tr>
<tr>
<td>Clinical isolate of <em>S. aureus</em> (<em>S. aureus</em> BM3002)</td>
<td>1082</td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em> ATCC# 14990</td>
<td>1082,782</td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em> ATCC# 12228</td>
<td>1082</td>
<td></td>
</tr>
<tr>
<td><em>S. saprophyticus</em> ATCC 15305</td>
<td>1082, 782</td>
<td></td>
</tr>
<tr>
<td><em>B. cenocepacia</em> CEP509</td>
<td>1082</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> PA01</td>
<td>1082</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em> H0142357</td>
<td>1082</td>
<td></td>
</tr>
<tr>
<td><em>A. baumannii</em> B0098426R</td>
<td>1082</td>
<td></td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em> H99 virulent</td>
<td>1082</td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em> ATCC# 90028</td>
<td>1082</td>
<td></td>
</tr>
</tbody>
</table>

**2.4.2 Minimum inhibitory concentration assay (MIC)**

MIC was done by using the bacterial or fungal culture in its mid-log phase diluted to 1/200 concentration. A master plate for drug was made containing 100x their final concentration in DMSO. The highest concentration of the stock was 6400 µg/ml and then diluted two-fold until the last concentration was 3.125 µg/ml. One microlitre of the drug stock was added to 99 µl of the media to give 1/100 dilution of the stock. One hundred microlitre of the 1/100 dilution of the indicator strains were added to the drug and incubated overnight at 37°C for bacteria and 30°C for fungi without shaking. MIC plates were read visually as well as on a plate reader at OD₆₀₀ for bacteria and OD₅₃₀ for fungi.
2.4.3 Edman degradation and sequencing of the compound 1082 [M+2H]^{2+}

The pure dried compound 1082 [M+2H]^{2+} was sent to the Hospital for Sick Children, Toronto for Edman degradation and sequencing.

2.5 Media Recipes

2.5.1 Maltose Yeast Malt (MYM)

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

Make in 1 L of 50% milliQ water and 50% tap water
Add 2 ml of R2 trace elements per liter of media after autoclaving

2.5.2 Mannitol Soy agar (MS)

Mannitol                      20 g  
Soy                          20 g  

Make in 1 L of milliQ water. After autoclaving add 1 ml of 1M MgCl$_2$ per 100 ml of MS.

2.5.3 SAM

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

Make in 1 L of milliQ water and autoclave.
Chapter 3: Results

3.1 Effects of a gain-of-function allele of a pleiotropic regulator of secondary metabolism on heterologous actinomycetes

AfsQ1 is one of the 28 known pleiotropic regulators of secondary metabolism identified in *S. coelicolor*. It is the response regulator of the two-component signal transduction system AfsQ1/Q2. The signal required to activate the expression of this system is unknown; however it is envisioned to play a role in triggering responses in diverse genes such as, biosynthetic gene clusters for secondary metabolites and variation in the availability of fixed nitrogen. This cascade effect is a consequence to phosphorylation of the response regulator component, AfsQ1, which is difficult to achieve in the absence of specific signal binding to the sensor kinase component, AfsQ2. The capability of AfsQ1, however, can be harnessed if a constitutively active form is constructed. For this purpose, a mutant allele of *afsQ1* was created by replacing aspartate, the site of phosphorylation (D52) with a glutamate residue, as such mutations often give rise to constitutively active response regulator. This mutant allele of *afsQ1*, *afsQ1*+, was cloned in the pSET152-*ermE*p vector. *ermE* is a constitutively active promoter of erythromycin-producing strain of *Saccharopolyspora erythraea*. In my work, pSET152-*ermE*p-*afsQ1*+ was introduced into wild-type environmental strains obtained from the Wright Actinomycetes Collection (WAC). For convenience, the vector containing the inserted gene *afsQ1*+ is abbreviated as “Q1*”, the empty vector containing the *ermE* promoter but no gene is abbreviated as “EV”, the vector containing the wild type *afsQ1* gene is abbreviated as “Q1” whereas the wild type strain is abbreviated as
“WT” throughout the thesis. In order to generate a collection of actinomycete strains expressing the Q1* allele, I used a medium-throughput conjugation protocol devised in our laboratory by Tomas Gverzdys\textsuperscript{104}. A donor strain of \textit{E. coli} (refer to Materials and Methods section) was used to introduce EV, Q1 and Q1* into 120 wild WAC isolates (materials and methods). All conjugations were carried out in triplicate with, in general, good reproducibility. Success of the conjugation was determined by the number of apramycin-resistant exconjugants.

Figure 6 depicts the results of conjugation in 120 WAC strains, none of which have been previously subjected to genetic modification. Starting with the control vector (EV) which contains the \textit{ermE*} promoter but no inserted gene, I found that, of 120 strains, I observed apramycin resistant exconjugants in 55 strains (46\%). Similarly, when I used the vector expressing the wild type Q1 gene, I observed exconjugants in 53 strains (44\%), most of which had also been modified with EV. In marked contrast, when I carried out a conjugation experiment with the Q1*-expressing vector I only observed exconjugants in 10 strains (8\%). Again, these strains were also modified by EV and Q1, however by looking at the number of exconjugants with Q1*-expressing vector, it is clear that the mutant \textit{afsQ1} gene was not well-tolerated by many strains.

These results illustrate two things. First, using the technology at our disposal, we could genetically modify 40-50\% of wild actinomycetes. We assume that the other strains are either resistant to this modification because of the restriction barriers or that they do not tolerate the \textit{ΦC31} integration mechanism. Second, the \textit{afsQ1*} gene appears to exert a toxic effect on many exconjugants, explaining the reduced frequency of exconjugants.
While it is difficult to ascribe any plausible basis for this, it suggests that the gene is in fact expressed at a level sufficient to exert a biological effect.

**Figure 6** - Percentage of exconjugants seen after conjugation of 120 WAC strains with EV (in red), Q1* (in green) and Q1 (in blue). Results indicate toxic effect of Q1* on the growth of the actinomycetes. Percentage is calculated as mean with error bars as standard deviation for three experiments.

### 3.1.1 Induction of antimicrobial activities by Q1*

Previous studies in our laboratory (Tomas Gverzdys, Daniel Socko, and Justin Nodwell, unpublished data) suggested that Q1* could induce or enhance secondary metabolism in *S. coelicolor* as well as one wild actinomycete isolate, WAC04657. Importantly, WAC04657 containing Q1* exhibited antimicrobial activity against resistant pathogens, namely *Pseudomonas aeruginosa* PAO1 (Figure 7) and *A. baumannii* B0098426R (figure not shown). This is interesting as both strains are typically very difficult to kill with known antibiotics.
Figure 7 - Colony diffusion assay of WAC04657 against *P. aeruginosa* PAO1. The strain containing Q1* shows higher antimicrobial activity as evident by a larger zone of inhibition as compared with the strain containing Q1 or the control WT and EV strains (Figure taken from Gverzdys, Socko and Nodwell, unpublished data).

Several WAC strains were selected to screen for their ability to produce antimicrobials, as previous work in our laboratory showed increased production of antimicrobial compounds after introduction of a modified pleiotropic regulator *lsr*$_{NTD}^{104}$. I explored the antibacterial activities of WAC00205, WAC00206, WAC00226, WAC00230, WAC00263 and WAC00290 expressing Q1* or containing the control EV. Preliminary screening of antimicrobial production by these genetically modified strains was done by colony diffusion assay against the indicator strains: *Escherichia coli* ATCC# 25922, *Bacillus subtilis* #168, or *Micrococcus luteus*. Antimicrobial activity was evident when there was a zone of inhibited growth of the indicator strain around the actinomycetes colonies.

This preliminary screening led to the selection of two strains, WAC00263 and WAC00226, for follow up experiments, since Q1* induced or enhanced the antimicrobial activity relative to the EV control in MYM and R2YE media. For convenience,
Exconjugants of WAC00263 and WAC00226 containing Q1* are referred to as “WAC00263::Q1*” and “WAC00226::Q1*”, respectively throughout the thesis. Similarly, exconjugants of WAC00263 and WAC00226 containing EV are referred to as “WAC00263::EV” and “WAC00226::EV”, respectively.

WAC00263::Q1* showed a larger zone of inhibition as compared with the WT and the EV control strains, when tested against the indicator Gram-positive bacterium *M. luteus* on MYM media (Figure 8) and R2YE media (figure not shown). The WT strain WAC00226, and strain containing the EV showed antimicrobial activity against *M. luteus* on MYM media but the activity was further increased by WAC00226::Q1* (Figure 9A). Similarly, on R2YE media, WAC00226::Q1* showed an increased zone of inhibition against *M. luteus* as compared to the control strains (Figure 9B). Against *E. coli* and *B. subtilis*, both WAC00263::Q1* and WAC00226::Q1* showed no significant increase (~1 mm) in the zone of inhibition in comparison with its WT and EV control strains.

Our results provide unequivocal evidence that the strains containing Q1* enhanced antimicrobial activity compared to the control strains in a wide array of WAC strains. Furthermore, it also suggests that despite the fact that introduction of Q1* gives fewer exconjugants, two out of the six selected WAC strains were those that increased antimicrobial production.
Figure 8 - Zone of inhibition of WAC00263 following bioassays performed on MYM media against *M. luteus*. Spores containing WAC00263 (WT), WAC00263::Q1*, and WAC00263::EV were spotted on MYM media and grown for 7 days before an overnight culture of indicator organism was overlayed on these plates. Zones of inhibition were measured on the 8th day of incubation. Increased zone of inhibition was seen around WAC00263::Q1* when compared with the control strains. Zone of inhibition is calculated as mean with error bars as standard deviation for three experiments. Left: quantification of the size of zone of inhibition; Right: colony diffusion assay.

Figure 9 - Zone of inhibition of WAC00226 following bioassays when performed against *M. luteus* on (A) MYM and (B) R2YE. Spores containing WAC00226 (WT), WAC00226::Q1*, and WAC00226::EV were spotted on different media and incubated for 6 days before an overnight culture of indicator organisms were overlayed on these plates. Zones of inhibition were measured on the 7th day of incubation. WAC00263::Q1* showed increased zone of inhibition against *M. luteus* on both the media conditions. Zone of inhibition is calculated as mean with error bars as standard deviation for three experiments. Left: quantification of the size of zone of inhibition; Right: colony diffusion assay.
3.1.2 Confirmation of the integration of the vector containing \(afsQ1^*\) and control (EV) into the exconjugants

The apparent toxicity of Q1* in many actinomycetes led to concerns that the gene might be unstable in some exconjugants. I therefore carried out an experiment to determine whether the mutant allele was in fact present in the strains that exhibited elevated, and apparently Q1*-induced antimicrobial activity.

pSET152 is an integration vector containing an \(attP\) site and \(int\) site derived from a \textit{Streptomyces} phage \(\Phi C31\) which encodes an enzyme integrase that mediates efficient integration between its own \(attP\) site and \(attB\) site in the bacteria\(^{105}\). The regulator \(afsQ1^*\) was cloned downstream of the strong constitutive promoter \(ermE^*\). Following conjugation, the EV or Q1* gets integrated into the chromosome. Primers were designed to hybridize to EV vector and amplify a region of size 565 bp. The molecular size of the gene Q1* is 675 bp. In case of exconjugants with vector containing the gene Q1* band of size 1.28 kb (565+675=1.24 kb) was expected.

Chromosomal DNA of WAC00263 and WAC00226 exconjugants were extracted and subjected to PCR using Q1*-specific primers as described in the Materials and Methods section. In WAC00263 and WAC00226, a band size of 1.24 kb was observed for DNA isolated from WAC00263::Q1* and WAC00226::Q1* and a band of size 565 bp was observed for the WAC00263::EV and WAC00226::EV (Figure 10), confirming that the vector containing the gene Q1* and the EV was successfully integrated into their respective genomes. The proof that the exconjugants had the expected gene integrated in
their chromosomes further supports the suggestion that the inserted gene Q1* was responsible for the antimicrobial activity seen in both WAC00263 and WAC00226.

![Figure 10](image.jpg)

**Figure 10** - PCR confirmation of the WAC00263 and WAC00263 ex-conjugants. A 1.2 kb amplicon indicates that the vector containing Q1* has been integrated into the genome in the strains WAC00263 (lane 2) and WAC00226 (lane 4), and amplicon of 565 bp indicates successful integration of empty vector into the same strains WAC00263 (lane 3) and WAC00226 (lane 5).

### 3.2 Characterization of the bioactive molecules induced in Q1*-expressing strains

#### 3.2.1 Comparative metabolic profiling of Q1* and control strains derived from WAC00263 and WAC00226

Preliminary screening for antimicrobial activity led to the identification of two strains, WAC00263 and WAC00226, which reproducibly exhibited increased antimicrobial dependence on Q1*. To determine the molecule(s) responsible for the
bioactivity expressed by the gene Q1* in WAC00263 and WAC00226, butanol extracts from both the strains were prepared and the extracts were subjected to liquid chromatography-mass spectrometry (LC-MS). In LC-MS analysis base peak chromatograms were analyzed, where the molecules present in the crude extract were seen as their mass-to-charge (m/z) ratio.

**Compounds 1082 [M+2H]^{2+} and 782 [M+H]^+ induced by Q1* in the strain WAC00263**

When WAC00263 was grown on R2YE media, seven molecules were seen to be increased and one molecule was repressed in the strain expressing Q1* as compared to its WT and EV controls (Table 6). In this particular study, we are most interested in antimicrobial molecules that are enhanced or induced by expression of Q1*. Out of the seven molecules, 782 [M+H]^+ was selected for further study as it was only induced when Q1* was expressed (Figure 11A). This raised an inspiring possibility of it being a product of a cryptic gene cluster. In addition, 782 [M+H]^+ was also seen to be increased by expression of Q1* in MS media (data not shown) suggesting that the regulator Q1* may be playing more than a media-dependent role in enhancing or inducing secondary metabolite production. Additionally, 942 [M-H]^− was also induced by Q1* expression in R2YE media but was not studied further in this thesis. Thus, it is one of the priority molecules for further study.
Table 6 – Secondary metabolites induced or repressed* by WAC00263::Q1* when grown on R2YE and MYM media and their respective fold changes when compared to WT or EV controls.

<table>
<thead>
<tr>
<th>Media</th>
<th>Mass-to-charge ratio (m/z)</th>
<th>Retention time (minutes)</th>
<th>Fold increase/Induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2YE</td>
<td>782.31 [M+H]^+</td>
<td>8.33</td>
<td>Induced</td>
</tr>
<tr>
<td></td>
<td>942.36 [M-H]^−</td>
<td>7.76</td>
<td>Induced</td>
</tr>
<tr>
<td></td>
<td>783.31 [M+H]^+</td>
<td>8.32</td>
<td>~6</td>
</tr>
<tr>
<td></td>
<td>256.13 [M+H]^+</td>
<td>7.15</td>
<td>~5</td>
</tr>
<tr>
<td></td>
<td>1082.41[M+H]^+</td>
<td>7.70</td>
<td>~4</td>
</tr>
<tr>
<td></td>
<td>798.33 [M+H]^+</td>
<td>11.93</td>
<td>~3</td>
</tr>
<tr>
<td></td>
<td>784.53 [M+H]^+</td>
<td>12.22</td>
<td>~2</td>
</tr>
<tr>
<td></td>
<td>895.41[M-H]^−</td>
<td>7.76</td>
<td>*~10</td>
</tr>
<tr>
<td>MYM</td>
<td>1082.31[M+2H]^2+</td>
<td>7.68</td>
<td>Induced</td>
</tr>
<tr>
<td></td>
<td>325.07 [M-H]^−</td>
<td>7.02</td>
<td>~2</td>
</tr>
</tbody>
</table>

*” indicates molecules repressed by expression of Q1*
Similarly, when WAC00263 was grown on MYM media, two molecules were seen to be increased in WAC00263::Q1* as compared to its WT and EV controls (Table 6). Interestingly, the molecule 1082 [M+2H]^{2+} was not seen in WT and EV control, but was induced by expression of Q1* (Figure 12A). Although this molecule was also produced in R2YE media, its production on MYM media was significantly higher. The dependence of production of 1082 [M+2H]^{2+} on Q1* led us to pursue its molecular
characteristics. It was of particular interest because no matches were found for the compound 1082 \([M+2H]^2+\) in the natural product database suggesting that it might be a novel product. Moreover, the mass spectrum analysis revealed that the molecule with the m/z value 1082 \([M+2H]^2+\) was doubly charged and thus the monoisotopic molecular weight of the molecule was deduced to be 2161.820 (Figure 13).

LC-MS analysis revealed several Q1*-dependent compounds. Streptomyces produce many different kinds of secondary metabolites only some of which exhibit antibacterial activity\(^\text{102}\) so it was difficult to predict which of these induced compounds was responsible for the antimicrobial activity observed in the colony diffusion assays. Therefore, it was important to confirm that the crude extract still contained these antimicrobial molecule(s). In order to determine this, I performed disk diffusion assays against indicator strains: \textit{B. subtilis} and \textit{M. luteus}. The disks containing the crude extracts from WAC00263 (WT), WAC00263::Q1* and WAC00263::EV were placed on the LB agar plates with the indicator organism which were then incubated overnight. Extracts collected from WAC00263::Q1* grown on R2YE media showed a zone of clearance against \textit{M. luteus} (Figure 11B) and \textit{B. subtilis} (figure not shown), whereas no zone was evident in the case of the WT and EV controls. Similar results were recorded from extracts obtained from WAC00263::Q1* grown on MYM media (Figure 12B).
Figure 12 - Effect of *afsQ1* overexpression on secondary metabolite production in WAC00263 on MYM media. (A) Base peak chromatogram of WAC00263 (WT) (in blue), strain containing EV (in green) and strain containing Q1* (in red). The number above the peaks indicate mass-to-charge ratio (m/z). A peak with m/z of 1082.43, was seen to be induced by the regulator *afsQ1* relative to WT and EV control. (B) Disk diffusion assay with crude extract of WAC00263 (WT), WAC00263::Q1* and WAC00263::EV grown on MYM media. WAC00263::Q1* showed antimicrobial activity against *M. luteus* and *B. subtilis* while WT and EV showed no activity. Negative control (solvent control) and Media control (MYM control) showed no activity indicating the activity was induced by the regulator Q1*. 
Figure 13 - Mass Spectrum of 1082 \([M+2H]^2+\) exhibits isotopic peaks that differ by 0.5 mass units, indicating doubly charged ions. Thus the calculated monoisotopic molecular weight of the same molecule is 2161.820.

Compounds induced by Q1* in the strain WAC00226

In the preliminary colony diffusion assays, we have seen that the WAC00226 (WT) demonstrated antimicrobial activity and that expression of Q1* increased its activity. Similar to these results, in the LC-MS analysis, secondary metabolites identified in the crude extract of WAC00226 (WT) were increased several fold on expression of the gene Q1*. WAC00226::Q1* showed enhanced production of molecules 329 \([M+H]^+\) by ~6 fold, while 371 \([M+H]^+\), 357 \([M+H]^+\) and 385 \([M+H]^+\) were each enhanced by ~3 fold (Figure 14). These molecules, which differed from each other by m/z value of 14, were seen to be eluted after one another and thus could be structurally related to each other. This can be confirmed by the NMR analysis in the future. Though these molecules were produced in the wild type strain, Q1* expression significantly increased their yield. These molecules have not yet been purified and characterized.
Figure 14 - Effect of Q1* overexpression on secondary metabolite production in WAC0026 on R2YE media. Base peak chromatogram of WAC0026 (WT) (in blue), strain containing EV (in green) and strain containing Q1* (in red). The number above the peaks indicates the mass-to-charge ratio (m/z) of the peaks. Overexpression of Q1* has caused increased production of five 300-series molecules by several fold and are seen to be eluting one after the other.

The LC-MS chromatograms for both WAC00263 and WAC00226 showed that diverse secondary metabolites were enhanced by Q1* in both strains. Furthermore, purification of the molecules recorded in the LC-MS chromatograms was carried out to determine if the increase in antimicrobial activity was due to these unknown molecules. In this work, I decided to focus on the purification of molecules with the m/z values of 1082 [M+2H]^{2+} and 782 [M+H]^+ from the strain WAC00263. This is because these molecules were not produced in WAC00263 (WT) and the WAC00263::EV in amounts sufficient to kill the indicator organisms, however, expression of Q1* in the same strain induced the production of these two molecules in exceedingly higher amounts that resulted in the killing of the indicator bacteria in the disk diffusion assays (10A1 and
10A2). The results raised an interesting possibility that these two molecules could be products of cryptic gene cluster activation, and that they could be novel compounds. The second strain, WAC00226 (WT) showed antimicrobial activity against the tested indicator organisms and with expression of the gene Q1*, we were able to increase the production of these molecules making it interesting for future studies.

3.2.2 Purification and antimicrobial testing of 1082 [M+2H]^2+ and 782 [M+H]^+

In order to purify the two molecules discussed above, large scale butanol extracts were prepared. The presence of the desired molecules 1082 [M+2H]^2+ and 782 [M+H]^+ was confirmed by LC-MS analysis and antimicrobial activity against *M. luteus* and *B. subtilis* was similarly verified by disk diffusion assays.

I purified 1082 [M+2H]^2+ from the crude extract using isocratic (50% acetonitrile) elution HPLC method. The compound 1082 [M+2H]^2+ was eluted at a retention time of 4.3 minutes. To check for purity, the sample 1082 [M+2H]^2+ was re-injected into the HPLC where the chromatogram showed a single peak (Figure 15). This compound showed absorbance at 278 nm, 203 nm and 225 nm (inset of Figure 15) consistent with the presence of aromatic and carbonyl groups.
Figure 15 - Re-injection of the purified compound 1082 [M+2H]^{2+}. The HPLC chromatogram has shown a single peak eluting at the retention time 4.3 minutes. The compound absorbs at 278 nm, 203 nm and 225 nm. U.V absorbance profile (overlaid on top of the chromatogram), indicating that the molecule is aromatic with carbonyl groups.

Furthermore, using disk diffusion assays, the pure compound 1082 [M+2H]^{2+} was screened for bioactivity against several test organisms such as *E. coli* ATCC# 25922, *B. subtilis* #168, *M. luteus* and *S. cerevisiae*, which represented different microbial groups (bacteria and fungi). This compound showed activity against *B. subtilis* #168 and *M. luteus*, while no activity was demonstrated with solvent control (Figure 16). The compound, however, did not show activity against *E. coli* ATCC# 25922, or *S. cerevisiae*.

The bioactivity of the compound 1082 [M+2H]^{2+} was further tested against 13 biosafety level-2 (BSL-2) pathogens, seven of which are multi-drug resistant (see Materials and Method section). Interestingly, the molecule 1082 [M+2H]^{2+} showed bioactivity against all the tested Gram positive bacteria – c-MRSA, VRE ATCC# 51299,
S. aureus, S. aureus BM3002, S. epidermidis ATCC# 14990, S. epidermidis ATCC# 12228 (Figure 17) and S. saprophyticus (not shown). The most striking observation was that this molecule was able to inhibit the growth of the Gram-negative bacterium A. baumannii B0098426R, an opportunistic pathogen known for significant antibiotic resistance. Interestingly, while the compound did not inhibit S. cerevisiae or Candida albicans, it was active against the virulent strain of fungus C. neoformans H99. From these results, it is apparent that the molecule 1082 [M+2H]^{2+} was active against diverse groups of microorganisms.

**Figure 16** - Disk diffusion assay with the pure compound 1082 [M+2H]^{2+} against laboratory strains. Compound 1082 [M+2H]^{2+}, purified using the extract collected from WAC00263::Q1* grown on MYM media and dissolved in DMSO, showed antimicrobial activity against (A) M. luteus and (B) B. subtilis. Negative solvent control DMSO showed no activity, indicating the activity was due to the molecule 1082 [M+2H]^{2+}.
**Figure 17** - Disk diffusion assays with the compound 1082 [M+2H]^{2+} against resistant pathogens. 1082 [M+2H]^{2+} purified using extract collected from WAC00263::Q1* that was grown on MYM media and dissolved in DMSO showed antimicrobial activity against (A) MRSA, (B) Clinical isolate of *S. aureus* BM3002, (C) *S. aureus*, (D) *S. epidermidis* ATCC# 14990, (E) *S. epidermidis* ATCC# 12228, (F) VRE ATCC# 51299, (G) *C. neoformans* virulent H99, and (H) *A. baumannii* B0098426R. Negative control DMSO showed no activity, indicating the activity was due to the molecule 1082 [M+2H]^{2+}.

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>1082 [M+2H]^{2+}</th>
<th>DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Clinical isolate of methicillin resistant <em>S. aureus</em></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>(B) Clinical isolate of <em>S. aureus</em> BM3002</td>
<td><img src="image3.png" alt="Image" /></td>
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<tr>
<td>(C) <em>S. aureus</em></td>
<td><img src="image5.png" alt="Image" /></td>
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<tr>
<td>(D) <em>S. epidermidis</em> ATCC# 14990</td>
<td><img src="image7.png" alt="Image" /></td>
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<tr>
<td>(E) <em>S. epidermidis</em> ATCC# 12228</td>
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</tr>
<tr>
<td>(F) VRE ATCC# 51299</td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>(G) <em>C. neoformans</em> virulent H99</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
</tr>
<tr>
<td>(H) <em>A. baumannii</em> B0098426R</td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
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</tbody>
</table>
In order for an antimicrobial drug to be clinically relevant it should be able to kill the target bacteria at a low concentration which is not toxic to the human cells. The simplest way to test the potency of a drug is by determining its minimum inhibitory concentration (MIC). Liquid MIC was done in a microtitre plate in duplicate. The highest concentration of the stock was 64 µg/ml which was diluted two-fold until the last concentration of 0.03125 µg/ml.

The MIC values for these bacteria are specified in the Table 7. The compound 1082 [M+2H]^{2+}, at concentrations as low as 1-8 µg/ml, was enough to kill Gram-positive bacteria such as *B. subtilis* #168, *M. luteus*, *S. aureus*, a clinical isolate of *S. aureus BM3002*, *S. epidermidis ATCC 14990*, and *S. saprophyticus ATCC 15305*, indicating that this molecule is quite potent. Interestingly, the compound 1082 [M+2H]^{2+} killed a virulent *C. neoformans* H99 at an MIC of 16-32 µg/ml. In the preliminary disk diffusion assays, the molecule was also active against VRE ATCC# 51299, c-MRSA, however it did not kill them at the highest concentration tested (i.e. 64 µg/ml). Such problem may be overcome by deploying a known antibiotic with 1082 [M+2H]^{2+} that can produce stronger effects when used in combination compared to when either of them was used alone.
Table 7 - MIC values of the compound 1082 [M+2H]^{2+} done in a microtitre plate in duplicate

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC values (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{B. subtilis} #168</td>
<td>2-4</td>
</tr>
<tr>
<td>\textit{M. luteus}</td>
<td>1</td>
</tr>
<tr>
<td>\textit{S. aureus}</td>
<td>8</td>
</tr>
<tr>
<td>\textit{S. aureus BM3002}</td>
<td>8</td>
</tr>
<tr>
<td>\textit{S. epidermidis} ATCC 14990</td>
<td>8</td>
</tr>
<tr>
<td>\textit{S. saprophyticus} ATCC 15305</td>
<td>32</td>
</tr>
<tr>
<td>c-MRSA</td>
<td>&gt;64</td>
</tr>
<tr>
<td>\textit{VRE ATCC# 51299}</td>
<td>&gt;64</td>
</tr>
<tr>
<td>\textit{C. neoformans} H99 virulent</td>
<td>16-32</td>
</tr>
<tr>
<td>\textit{A. baumannii} B0098426R</td>
<td>16-32</td>
</tr>
</tbody>
</table>

Structural characterization of the potent antimicrobial compound 1082 [M+2H]^{2+}

Tandem MS (performed by McMaster Regional Centre for Mass Spectrometry) revealed that the molecule is a peptide. In order to determine the amino acids in the peptide, Edman degradation and sequencing were carried out (Hospital for Sick Children, Toronto). Edman sequencing provided an 11 amino acid residue sequence which is as follows:

\text{Ser}_1\text{-Tyr}_2\text{-Ala}_3\text{-Ile}_4\text{-Val}_5\text{-Val}_6\text{-Ala/Phe}_7\text{-Ala/Phe}_8\text{-possibly Cys or a modified amino acid}_9\text{-Gly/Ala}_10\text{-Asp}_{11}.
The sequencing was carried out twice, independently, and it revealed that the sequencing process was arrested somewhere beyond the aspartate residue at position 11. A tBLASTn search of the NCBI DNA sequence database, which includes at least 70 annotated streptomycetes, did not reveal any hits, further suggesting that this compound is novel, and has the potential for future exploitation.

**Purification of the second unknown molecule 782 [M+H]+**

The compound 782 [M+H]+, as mentioned previously was produced in the strain WAC00263::Q1* on R2YE media. This was purified using the gradient elution HPLC method on a reverse-phase C18 column. Preliminary HPLC runs of the crude extract showed a large number of unresolved peaks (Figure 18A), making it difficult to separate the molecule. To overcome this problem, the crude extract was pre-fractionated on a reverse phase column, employing different solvent compositions to determine the concentration of the solvent at which the molecule is being eluted. The LC-MS of the fractions collected showed that the molecule with m/z 782 [M+H]+ was eluted at 75% MeCN + 25% water and 100% MeCN (see Materials and Methods). Finally, the compound 782 [M+H]+ was separated using the gradient of 50% to 90% acetonitrile and was seen to be eluted at retention time of 6.1 minutes (Figure 18B). The purity of the compound 782 [M+H]+ was checked by re-injecting it into the HPLC, where the chromatogram showed a single peak (Figure 19). Also, since the compound absorb at 193 nm, 254 nm and 280 nm (inset of Figure 19), it is likely to be an aromatic compound with carbonyl groups.
Figure 18 - Purification of the compound 782 [M+H]^+. (A) HPLC chromatogram of the injection of the crude extract of WAC00263::Q1* grown on the R2YE media. The chromatogram shows a large number of unresolved peaks. (B) HPLC chromatogram of the fraction injected using a new gradient, of the crude extract of WAC00263::Q1* grown on the R2YE media, showing a resolved peak for the 782 [M+H]^+ compound.
Figure 19 - Re-injection of the purified compound 782 [M+H]^+. The chromatogram shows single peak eluting at the retention time 6.1 minutes. U.V absorbance profile (overlaid on the figure), indicates that the molecule is an aromatic with carbonyl groups, as the compound absorbs at 280nm, 254nm and 193 nm.

The pure compound 782 [M+H]^+ was screened for bioactivity against laboratory strains such as *E. coli*, *B. subtilis*, *M. luteus*, and *S. cerevisiae* as well as Gram-positive BSL-2 pathogens such as *S. epidermidis* ATCC 14990 and *S. saprophyticus* ATCC 15305. The compound 782 [M+H]^+ showed activity against the Gram-positive strains such as *B. subtilis* and *M. luteus* while no activity was detected with the control DMSO (Figure 20). Additionally, 782 [M+H]^+ could not inhibit *E. coli*, or *S. cerevisiae*, *S. epidermidis* ATCC 14990, and *S. saprophyticus* ATCC 15305 (data not shown).

In order to check the potency of the compound, MIC values were determined against Gram-positive lab strains *B. subtilis* #168 and *M. luteus*, and a few additional BSL-2 pathogens such as e-MRSA, VRE ATCC# 51299 and *S. aureus*. The MIC was performed by using the same protocol as used for the molecule 1082 [M+2H]^2+. The
Figure 20 - Disk diffusion assay with the compound 782 [M+H]^+ against laboratory strains. 782 [M+H]^+, purified using the extract collected from WAC00263::Q1* grown on R2YE media and dissolved in DMSO, showed antimicrobial activity against (A) M. luteus and (B) B. subtilis. Negative solvent control DMSO showed no activity, indicating the activity was due to the molecule 782 [M+H]^+.

highest concentration of the molecule tested was 128 µg/ml and then diluted two-fold until the last concentration of 0.0125 µg/ml was obtained. The molecule 782 [M+H]^+ killed M. luteus at the highest concentration tested i.e. 128 µg/ml but did not kill B. subtilis even at the highest concentration. Moreover, the compound 782 [M+H]^+ did not inhibit any of the tested strains such as c-MRSA, VRE ATCC# 51299 and S. aureus even at the highest concentration. Though 782 [M+H]^+ is an antibiotic, it does not seem to be as potent as the molecule 1082 [M+2H]^{2+}. 
Chapter 4: Discussion

The overall goal of the research presented here was to activate cryptic secondary metabolite gene clusters by heterologous expression of a pleiotropic regulator from *S. coelicolor* and look for novel antimicrobial production. We were specifically focused on the discovery of antimicrobial compounds. One of the primary hurdles in activating the cryptic gene cluster is to activate the specific signal, which in most of the cases, is not known. I sought to bypass this need by genetically modifying the response regulator (RR) component of the two component signal transduction system (TCS) by making it constitutively active. To be specific, I selected AfsQ1, a RR belonging to TCS family, since it is broadly conserved in the streptomycetes. AfsQ1 is inactive unless it is phosphorylated, therefore, we created a mutant allele of *afsQ1*, *afsQ1*\(^*\), wherein the conserved aspartate residue which gets phosphorylated was substituted with glutamate, as such mutations often give rise to a constitutively active RR. The hypothesis of this study was that if the mutant allele is successfully inserted into wild isolates of actinomycetes, it would be possible to unlock cryptic genes, resulting in the biosynthesis of new molecules or enhanced production of low-level-expressed secondary metabolites. Introduction of mutant allele *afsQ1*\(^*\) in 120 WAC strains resulted in fewer ex-conjugants as compared to those with the EV control. Although we do not know the reason for this, it can be due to the fact that *afsQ1*\(^*\) is constitutively active and hence turning on the expression of antibiotics throughout the life cycle which, in turn, is making the cells sick.

A total of six strains were screened for Q1\(^*\)-induced antimicrobial production. Based on this screen, two strains WAC00263 and WAC00226, were identified in which
expression of the regulator Q1* showed enhanced antimicrobial activity. Although Q1* has served the purpose of inducing the production of antimicrobial molecules, its toxic effect on the cells makes it difficult to introduce into a large number of strains. To overcome this problem, a regulatable system is desirable. For example, the putative gene could be expressed under the control of an inducible promoter. To date, most of the inducible promoters that can be used in streptomycetes are triggered by antibiotics, which would make screening for antibiotic molecules very challenging. For example, the tipA promoter from S. lividans 66 is an inducible promoter whose signal is the antibiotic thiostrepton\textsuperscript{106}. This system can be used for screening of secondary metabolites which are not antimicrobials. Despite the potential toxicity, afsQ1* appears to be a good candidate pleiotropic regulator for inducing antibiotic production.

The usefulness of heterologous expression of the conserved pleiotropic regulators in diverse actinomycetes proved successful in expressing new antimicrobial / secondary metabolite molecules in the WAC strains. The most interesting result was the expression of antimicrobial compounds with m/z values of 1082 [M+2H]\textsuperscript{2+} and 782 [M+H]\textsuperscript{+} in the strain WAC00263. These compounds were induced only when the Q1* mutant allele was expressed heterologously.

In particular, the molecule 1082 [M+2H]\textsuperscript{2+} was identified to be an antimicrobial peptide that exhibited activity against a variety of bacteria as well as pathogens. The striking effects were on some of the multidrug resistant bacteria such as VRE ATCC# 51299 and clinical isolate of MRSA, which are naturally resistant to β-lactam antibiotics\textsuperscript{107}. Additionally, it was active against an opportunistic pathogen A. baumannii.
B0098426R and a virulent strain of fungus *C. neoformans* H99 but not active against laboratory strains such as *E. coli* ATCC# 25922 or *S. cerevisiae*. Though it is difficult to provide a reason behind these observations, it would be extremely exciting to investigate its mode of action against *A. baumannii* and *C. neoformans*. Although the compound demonstrated activity against *A. baumannii*, it was quite weak (the MIC value was 16-32 µg/ml and a hazy zone of inhibition was seen in the disk diffusion assay). There are alternate strategies available to fortify the antimicrobial potency against specific bacteria in case the MIC of the molecules is high. For example, the novel compound 1082 [M+2H]^{2+} obtained in the present study could be combined with silver, which has shown to enhance the activity of existing antimicrobials against Gram negative bacteria.\(^{108}\)

The second cryptic compound, 782 [M+H]^+, was found to be an antimicrobial with activity only against the Gram-positive bacteria *M. luteus* and *B. subtilis*. Unlike the potent molecule 1082 [M+2H]^{2+}, this molecule did not kill any of the Gram-positive multidrug resistant pathogens tested. Future work would involve testing it against other resistant Gram-negative pathogens such as *A. baumannii*, *P. aeruginosa*, *B. cenocepacia* and *K. pneumoniae*. Also, 782 [M+H]^+ by itself does not appear to be a potent antibiotic, as the MIC required to kill *M. luteus* was very high and the molecule did not show any inhibitory activity against *B. subtilis* even at the highest concentration tested. In order to improve the potency of this compound, further work could be done by looking for its synergistic partners. Though Q1* expression in the wild type strain increased the production of 782 [M+H]^+, the total yield was still very low. This problem can be
improved by expressing its biosynthetic gene cluster in chassis strains of *S. coelicolor* and *S. avermitilis*\textsuperscript{109,110}.

In addition to the antimicrobial compounds, 1082 \([M+2H]^2+\) and 782 \([M+H]^+\), which were produced in more than one media, several other media-specific Q1*-induced secondary metabolites were observed in the strain WAC00263. It would be very interesting to investigate if these molecules also have antimicrobial properties. This will help determine if Q1* is only involved in regulating the production of several antimicrobials or has its effect on other secondary metabolites such as, metal binding siderophores, spore pigments, signaling compounds and volatile odour compounds\textsuperscript{20}. In this study, the production of five 300-series molecules production was enhanced by heterologous expression of Q1* in the strain WAC00226. However, these molecules were not further isolated and studied. Therefore, in the future, the research could focus on purifying and characterizing the nature of these secondary metabolites. Interestingly as against Q1*-dependent increase in production of seven molecules in WAC00263 on R2YE media, only one molecule was repressed by Q1*. This suggests that Q1* is mainly involved in the activation of secondary metabolites.

Different organic solvents such as n-hexane, butanol, chloroform, acetone and ethyl-acetate could extract secondary metabolites from *Streptomyces*\textsuperscript{111}. In this study, butanol was used for all the crude extract preparations. Molecules are extracted in organic solvents based on their polarity. In the future, extracts employing different solvents could be compared by LC-MS. This may lead to the detection of more molecules like 1082 \([M+2H]^2+\) and 782 \([M+H]^+\), if any, induced by Q1* but not extracted in butanol.
In this work, I was able to provide evidence that the simple technique of heterologous expression of conserved pleiotropic regulators could be used as a tool to induce expression of novel cryptic antimicrobial molecules. The regulatory gene \textit{afsQ1}* induced production of an antimicrobial peptide molecule 1082 [M+2H]$^{2+}$. Tandem MS revealed that the molecule is a peptide. Edman degradation yielded 11 amino acids residues sequence from the N-terminal - Ser$_1$-Tyr$_2$- Ala$_3$-Ile$_4$-Val$_5$-Val$_6$-Ala/Phe$_7$- Ala/Phe$_8$-possibly Cys or a modified amino acid$_9$-Gly/Ala$_{10}$-Asp$_{11}$. Searches in the public database such as NCBI, natural product dictionary revealed no matches thus far, indicating that the molecule is novel. Further work is required to completely characterize the structure of the molecule.

There are several possible reasons for the arrest of the sequencing process beyond the aspartate residue beyond position 11 during Edman degradation. Edman degradation reactions are carried out by applying the sample onto the PVDF membrane. One of the possibilities behind incomplete residue analysis could be that the compound 1082 [M+2H]$^{2+}$ is adsorbing inefficiently onto this membrane, hence the starting molecule amount would not be sufficient to undergo the complete degradation cycle. Secondly, a more interesting possibility is that after the 11$^{th}$ residue, the molecule contains non-proteinogenic or modified amino acids like cystine and lanthionine. Lanthionine rings or modified amino acids cannot be detected by Edman reaction$^{112}$. Lantibiotics are lanthionine-containing peptide antibiotics, produced by large number of \textit{Streptomyces} and are well studied due to its role in the food industry for making cheese, and as food preservatives$^{113-115}$. In such cases, chemical modification can render it susceptible to
complete Edman degradation$^{112,116}$. In the future, this technique can be applied on the compound 1082 [M+2H]$^{2+}$ prior to Edman sequencing to hopefully get the full peptide sequence. Nevertheless, since we know 11 amino acid residues, we could still elucidate the biosynthetic cluster in the sequenced genome by looking for the nucleotides sequence corresponding to this amino acid sequence. Current efforts include subjecting chromosomal DNA from this strain to high throughput sequencing and identifying the candidate biosynthetic cluster.

Many strategies have been applied for improving secondary metabolite production, including conventional approaches such as manipulation of nutrient conditions, stress and random mutagenesis (Table 1). Also, several other recent genome mining techniques such as genome isotopic approach, gene knockout and comparative metabolic profiling have been used (Table 1). Although these techniques have been fruitful in activating secondary metabolite production, the majority of these require genome sequence analysis, substrate prediction, or they are random and unpredictable$^{57,58,61,65,67}$. An exception is a technique involving use of synthetic small molecule probes that do not require any genetic engineering of the strain but was found to be biased towards production of polyketides$^{102}$. We selected the strategy of heterologous expression for obvious reasons. An advantage of heterologous expression of pleiotropic regulators is that we can alter secondary metabolism in diverse actinomycetes strains without any prior knowledge of the regulatory system in the strains$^{76}$. Although the regulators involved in antibiotic production can vary between diverse streptomycetes, the overall mechanism is most likely conserved and hence is not random. Additionally, since
pleiotropic regulators are global regulators, they usually bring overall changes in the secondary metabolite production.

Using a similar technique, our lab was successful in inducing expression of the cryptic molecule pulvomycin in *S. flavopersicus*\textsuperscript{77}. Other studies have also been carried out using the same technique, where heterologous expression of the pleiotropic regulators *metK* and *afsR* from *S. peucetius* into *S. venezuelae* led to the increased production of the antibiotic pikromycin by 1.6-fold and 2.6-fold, respectively\textsuperscript{89}. Expression of another global regulator, the catabolite repressor protein (crp) from *S. coelicolor*, into diverse streptomycete strains significantly affected the production of antimicrobial molecules. Most interestingly, in the wild strain *S. sp.* SPB74, heterologous expression of *crp* increased production of two secondary metabolites by >22 and ~33 fold when compared to the control strains\textsuperscript{46}. Pharmaceutical industries are always working towards finding new ways to increase antibiotic production, and the use of such an approach could lead to significant gains in its yield.

There are however a few limitations that need to be taken into consideration while seeking to employ this technique. Firstly, the *Streptomyces* strains should not be resistant to apramycin. In this study, the plasmid pSET-erm\textsuperscript{E} being conjugated contains the apramycin resistance cassette. Because apramycin was used to select against the unsuccessful exconjugants, the strains with intrinsic resistance to apramycin cannot be used. Such strains can be initially screened out by growing them in the presence of apramycin. In our case, this did not seem to pose a problem with the wild strains used in this study. For strains with intrinsic resistance to apramycin, this problem can still be
overcome by using a different antibiotic selection marker. Secondly, since the introduction of the plasmid into the strains is by conjugation, the strain should be easily susceptible to intergeneric conjugation\textsuperscript{76}. In this study, introduction of the plasmid using medium-throughput conjugation was successful in 46\% of the strains. This can be attributed to the \textit{Streptomyces} restriction system or the lack of the target integration site in the genomes of these wild strains. Thirdly, it is important that the regulator used is conserved between diverse strains, since it increases the probability of directly influencing secondary metabolism when being heterologously expressed. Regulators such as \textit{crp} and \textit{afsQ1} are conserved across large number of streptomycetes, and their heterologous expression in wild soil actinomycete isolates has revealed its universal role in stimulating secondary metabolite production.

Regardless of the limitations presented here and the future work that still needs to be done on the structure of the two molecules, my work has demonstrated that this approach has tremendous potential in mining for novel antibiotics from wild strains. Using only one modified pleiotropic regulator, I was able to observe expression of several new secondary metabolites in diverse wild isolate of actinomycetes. The scope of employing this technique is immense considering that there are 28 pleiotropic regulators reported in \textit{S. coelicolor} alone\textsuperscript{102}. It is expected that our approach could be of wide use in unlocking the vast treasure of such compounds that nature has provided to these organisms.
Chapter 5: Conclusion

The future of the pharmaceutical industry mainly depends on the discovery of novel products from natural sources. Genome sequencing and analysis have shed light on the potential of actinomycetes to produce natural products that have not been exploited yet. Rapid discovery and understanding of the secondary metabolite gene clusters provide insight into their regulatory genes for the discovery of the novel compounds. We have proved the utility of heterologous expression of pleiotropic regulators as a potential tool in discovering novel antibiotics. By mining as few as six wild actinomycete isolates, which were heterologously expressing one of the 28 pleiotropic regulators, afsQ1*, in S. coelicolor we have demonstrated enhanced production of antimicrobials in two wild actinomycete strains. The most promising compound is a potentially novel potent antimicrobial peptide, 1082 [M+2H]^2+, which was active against diverse groups of microorganisms. Moreover, further characterization of the secondary metabolites enhanced by afsQ1* expression, which were not studied in this work, may provide insight in its role in regulation of non-antimicrobial secondary metabolites. The expression of the pleiotropic regulator afQ1*, though potentially toxic, has proven to be a useful tool to activate cryptic genes and produce novel antimicrobials. This regulator can further be exploited by expressing the regulator using a regulatable system in a wide array of well-studied Streptomycetes and also wild soil isolates to overcome its toxic effect. Nevertheless, we were able to provide evidence that this technique can be used effectively to induce the production of novel antimicrobials. The discovery of the novel compound
1082 [M+2H]$^{2+}$ and uncharacterized compound 782 [M+H]$^{+}$ opens a new avenue of study – the characterisation of the structure of compound and determining its mode of action.
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