ACTIVATION OF ACTINORHODIN EXPORT AND RESISTANCE IN STREPTOMYCES
A TWO STEP MECHANISM FOR ACTIVATION OF
ACTINORHODIN EXPORT AND RESISTANCE IN
STREPTOMYCES COELICOLOR

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TITLE: a two step mechanism for activation of actinorhodin export and resistance in *Streptomyces coelicolor*

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Abstract

Antibiotic producing organisms often bear cognate export and/or resistance mechanism encoded in the same cluster of antibiotic biosynthetic genes, but how expression of these protective genes associated with the cognate biosynthetic machinery was poorly understood. This work focuses on elucidating the mechanism by which the antibiotic producing bacteria initiate and sustain self-resistance in individual cells and across the colonies. Actinorhodin is a blue pigmented antibiotic produced by Streptomyces coelicolor. Within its biosynthetic gene cluster, an actAB operon encodes two putative efflux pumps and is regulated by the transcriptional regulator ActR. This work (1) identified that normal yields of actinorhodin require actAB expression and both actinorhodin and its 3-ring pathway intermediate (e.g. DHK) could relieve actAB expression from ActR repression in vivo; (2) created an ActR mutant that interacts productively with a 3-ring intermediate (e.g. (S)-DNPA) but not actinorhodin and responds to actinorhodin biosynthetic pathway with induction of actAB and normal actinorhodin production; (3) demonstrated that actinorhodin producing cells could induce actAB expression in non-producing cells and actinorhodin is the intercellular signal in this case; (4) showed actAB expression in “intermediate only” ActR mutant is short lived and in the absence of the actinorhodin-mediated signaling step the culture experiences widespread cell death. Based on these results, we proposed a two-step model for actinorhodin export and resistance where intermediates trigger initial expression for export from producing cells and actinorhodin then triggers sustained export gene expression that confers culture-wide resistance.
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Abbreviations

ABC: ATP-binding cassette
ACT: actinorhodin
ACP: acetyl carrier protein
ATP: adenosine triphosphate
CD: Circular Dichroism
CLF: chain length factor
DBD: DNA binding domain
DDHK: deoxy- dihydrokalafungin
DHK: dihydrokalafungin
DMAC: 3,8-dihydroxy-1-methylantraquinone-2-carboxylic acid
DMSO: dimethylsulfoxide
DNA: deoxyribonucleic acid
EMSA: electrophoretic mobility shift assay
FAD: flavin adenine dinucleotide
eGFP: enhanced green fluorescent protein
GTP: guanosine triphosphate
HACD: β-hydroxyacyl CoA dehydrogenase
HBC: 4-hydroxy-2,2’-bipyrrole-4-carbaldehyde
HBM: 4-hydroxy-2,2’-bipyrrole-4-methanol
kb: kilobase pair
kD: kilodalton
KR: ketoreductase
KS: keto-synthase
LBD: ligand binding domain
MBC: 4-methoxy-2,2’-bipyrole-5-carbaldehyde
NADH: reduced nicotinamide adenine dinucleotide
NRPS: non-ribosomal peptide synthase
ORF: openreading frame
PCP: Peptidyl Carrier Protein
PCR: polymerase chain reaction
PKS: polyketide synthase
PLP: pyridoxal phosphate
red: undecylprodigiosin
RNA: ribonucleic acid
RT-PCR: reverse transcription polymerase chain reaction
(S)-DNPA: 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-H-naptho-[2,3-c]-pyran-3-(S)-acetic acid
spp.: species
TFR: TetR family regulator
Tris: tris-(hydroxymethyl) aminomethane
UP: 2-undecyl-pyrrole
whi: white
Publications:


3 Xu Y, Willems A, Au-yeung C, Nodwell JR. *A two-step mechanism for the activation of actinorhodin export and resistance in Streptomyces coelicolor* accepted by mBio

Chapter I

Introduction

Adapted from

Xu Y, Nodwell JR. *Antibiotic resistance in antibiotic producing bacteria.*
Manuscript in preparation
1.1 The rise of antibiotics and the emergence of antibiotic resistance

The history of exploiting microorganisms can be traced back long before their discovery. The ancient Chinese distilled alcoholic spirits from fermented grains and the Egyptians of the Pharaohric era utilized carbon dioxide generated by brewer’s yeast for leavening dough. These empirical practices of microbiology were passed down for generations and still serve us today. Although the first images of these microscopic organisms were captured by Antonie Van Leeuwenhoek in the 17th century, they were not accepted as "a form of life" until a century later when Louis Pasteur and John Tyndall demolished the old concept of “spontaneous generation” and established the idea that these microbes are from pre-existing life forms. This milestone opened a window to the field of modern microbiology. Later, observations of antagonistic effects of microbes over one another raised huge interests for their therapeutic potential which quickly developed into the intentional large-scaled production of bio-products. In 1929, Alexander Fleming separated an active fluid from the *Penicillium notatum* liquid culture that killed the bacterium *Staphylococcus aureas* and named it penicillin. Facilitated by intense development of microbial genetics starting in the 1940’s, mutated high-yielding strains turned this laboratory curiosity into an enormous industrial success. Finally, penicillin was first used to treat wounded troops during World War II. In 1943, streptomycin, an aminoglycoside antibiotic produced by the soil bacterium *Streptomyces griseus* was discovered in Selman Waksman’s lab. It was found to have extensive application in treating a variety of infectious diseases including pulmonary tuberculosis.
Discoveries of penicillin and streptomycin and their commercial success initiated a
golden-era of antibiotics, the influence of which continues to the present day.

A boom in industrial microbiology brought the discovery of a large number of
microbial secondary metabolites with importance to human health. Like penicillin and
streptomycin, these structurally diverse bioactive molecules are not essential for cell
growth and are considered as resultant products from morphological differentiation
subsequent to unbalanced growth. They are supposed to improve the survival of the
producing organisms or to maintain their superiority in competition with other organisms.
They are multi-functional in nature, serving as mineral scavengers (e.g. siderophore),
signaling molecules that respond to stringent situations, or biological warheads aimed at
competitors in the same habitat\textsuperscript{2}. For years, the potency of killing or inhibiting other
microbes has attracted much of our attention. Antibiotics, as their name says, are agents
“against life”. These natural, semi-synthetic or fully synthetic molecules can selectively
stop essential processes in microbes (bacteria and fungi, some parasites, even mammalian
cells) and have been widely applied to our life. Over 50,000 tons of these molecules are
produced annually and we have benefited from these remarkable “wonder drugs” for over
60 years\textsuperscript{3}.

However, significant antibiotic-resistance developed by non-producing organisms
including pathogenic bacteria usually occurs in quick succession of the clinical
introduction of a new drug. The wide use of antimicrobial agents in medicine and
agriculture during the past 70 years has catalyzed the adoption of immunities against the
selective stress by many pathogens\textsuperscript{3}. In addition, returns from intense conventional
screening for new drugs are diminishing, as the antibiotic industry is frustrated by the rediscovery of the same compounds. The golden-era of antibiotic discovery has faded, but the needs for treatment of infectious diseases and the rapid development of multi-drug resistance set us on the horns of a dilemma. To break through, medicinal chemists modified the existing chemicals, most being natural products, in attempts to regain their potency against resistant microbes. These semi-synthetic compounds have made evident improvements: for $\beta$-lactam antibiotics, chemical modifications were introduced into structures of penicillin and cephalosporin, leading to multiple generations of new drugs with broader spectrum$^{3,5}$. Other parallel approaches for identifying new drugs include the continued screening of diverse microbial culture, combinatorial biosynthesis of antibiotics and expansion of synthetic chemical libraries with increasing architectural complexities$^9$. Application of metagenomics by recovering genetic materials directly from environmental samples hopes to explore genomes of drug producers that are uncultruable under current lab conditions for novel antibiotics. However, none of them has given us prosperous findings so far. Based on the observations that genes encoding secondary metabolic pathways are clustered together in bacteria, researchers are now trying to manipulate these cryptic pathways that are predicted to synthesize bioactive compounds but are silent or expressed at very low level under regular lab conditions.

1.2 Resistance/export mechanisms in antibiotic producing bacteria

The fact that many resistance determinants in antibiotic non-producers reside on mobile genetic elements, such as transposons or plasmids, suggests that antibiotic
resistance is moved among microbes by horizontal gene transfer and sheds a light to its origin: antibiotic producers that harbour self-resistance genes to avoid suicide\(^6, 7\). The same type of resistance genes were identified among bacteria that produce the same functional class of antibiotics. For example, some aminoglycoside antibiotics, such as kanamycin and gentamicin, inhibit bacterial protein synthesis by reversibly binding with the 30S subunit of ribosome\(^8, 8-11\). The self-resistance gene, \textit{kmr}, in the kanamycin producer \textit{S. kanamyceticus} encodes an rRNA methylase that modifies the 16S rRNA of 30S ribosomal subunit and has widespread homologues among other aminoglycoside producers, mostly in the Gram-positive \textit{Streptomyces} genus and \textit{Micromonospora} genus\(^9-11\). Since the soil provides a highly competitive and complicated micro-environment where killing and survival are routine, a mighty antibiotic producer like \textit{Streptomyces} is also a source of a “resistome” that antibiotic non-producing organisms in the same niche would have likely co-evolved with\(^12, 13\). An extensive investigation was carried out on soil-dwelling bacteria, \textit{Streptomyces} spp., so far the biggest reservoir of bioactive molecules, consisting of a screen of 480 \textit{Streptomyces} isolates against 21 antibiotics. This effort revealed that most strains were resistant to, on average, seven to eight antibiotics including many antibiotics not produced by the resistant strains\(^13\). The implication is that antibiotic resistance genes that originate in the producers are transferred to other organisms where they are amplified through Natural Selection.
Figure 1.1 "Target based" and "access based" antibiotic resistance strategies

By target based strategies, the antibiotic target is protected by either covalent modification (e.g. methylation on 16S rRNA); synthesis of intrinsic resistant target or switch to a resistant copy (e.g. intrinsic resistant RNA polymerase or resistant version of gyrase B subunit); or target binding protection protein (e.g. Ribosomal Protection Proteins). By access based strategies, the access to the target is cut off by temporal inactivation or sequestration of the drug. In addition, efflux pumps not only transport drugs cross the cell membrane, but confer resistance by reducing intracellular accumulation of toxins as well.

There have been numerous reviews on self-resistance mechanisms in antibiotic producing bacteria (Figure 1.1)\textsuperscript{6,7}, but none has given a comprehensive discussion on the relationships between production and resistance. As antibiotic production is not essential for cell growth, resistance to these secondary metabolites needs to be tightly regulated and expressed concomitantly with production. It seems to be a genetic evolutionary arrangement that resistance genes are usually clustered with relevant antibiotic biosynthetic genes and such proximity facilitates coordinated regulation of the two. Although prediction of transcriptional units was not carried out for all the known
biosynthetic clusters and functional studies on many of these biosynthetic gene clusters are incomplete, there are several informative examples indicating direct coordination between biosynthesis and resistance. To introduce the topic of my PhD thesis, I will discuss several of the best understood mechanisms.

1.3 Transcriptional association between resistance and biosynthesis

1.3.1 Oxytetracycline/tetracycline

Oxytetracycline is a tetracycline antibiotic (Figure 1.2A). Members of this group (e.g. tetracycline, oxytetracycline and chlortetracycline) interfere with the elongation phase of protein biosynthesis by blocking the interaction of aminoacyl-tRNAs with the A site of the ribosome primarily through an interaction with the irregular minor groove of helix34 and the loop of helix31 in the 16S rRNA of the 30S subunit. This turns protein biosynthesis into a non-productive phase as peptide bonding and translocation are abolished (Figure 1.2B)\textsuperscript{14,15}.

The known resistance strategies against tetracyclines include: (a) efflux-mediated reduction of intracellular antibiotic accumulation\textsuperscript{16-18}, (b) enzymatic degradation\textsuperscript{19,20} and (c) rRNA mutations\textsuperscript{21}. However, ribosomes isolated from some tetracycline producers (e.g. \textit{Streptomyces aureofaciens}) were sensitive to the drug, implying a factor other than covalent modification during protein synthesis is responsible for the resistance\textsuperscript{14}. Ribosomal protection protein (RPP) mediated tetracycline resistance was first described in 1985 by a Japanese group who identified two tetracycline resistance genes from the oxytetracycline producer \textit{Streptomyces rimosus}\textsuperscript{22}: \textit{otrB} was found responsible for reduced
intracellular accumulation of oxytetracycline, and *otrA* directed resistance resided mainly in crude ribosomes and was lost when washed with 1 M ammonium chloride\(^22\). Its gene product was characterized as “a cytoplasmic factor” that confers tetracycline resistance to the protein biosynthetic machinery not through covalent modification of the ribosomal subunit\(^{22, 23}\). Orthologues of OtrA (Tet(O), Tet(M), *etc.*) were identified in clinical pathogens and these RPPs belong to the GTPase superfamily and are all similar to elongation factors (e.g. EF-Tu and EF-G)\(^{14, 15}\). Model studies of Tet(O) showed that RPPs interacted with helix18/34 of the 16S rRNA and dislodged tetracycline from the ribosome, meanwhile decreasing the binding affinity of tetracycline to the ribosome (Kd of tetracycline binding to ribosome increased from 5 μM to 30 μM)\(^{14, 15}\). These proteins then hydrolyzed their bound GTP, dissociated from the ribosome and the ribosome returned to its regular elongation cycle (Figure 1.2B)\(^{14, 15}\).

Oxytetracycline biosynthetic genes are clustered and flanked by resistance genes *otrA* and *otrB*, covering 30 kb of chromosomal DNA (Figure 1.2C)\(^{24}\). Assembly of the tetracycline backbone follows a type II iterative polyketide synthetic pathway and the nascent polyketide is cyclized and modified to give mature oxytetracycline. Regulation of resistance genes is well coordinated with antibiotic production. *otrB*, encoding the efflux pump, is activated by the adjacent regulatory gene *otrR*, which mirrors the “tetR-tetA” system that I will discuss in section 1.5. Transcription of *otrA*, the RPP gene, is switched between two promoters: *otrAp1* and *otcCp1* (Figure 1.2C)\(^{23}\). During exponential growth, *otrA* is transcribed as a single cistron from *otrAp1*. Once the bacterium enters stationary
growth phase and begins to synthesize oxytetracycline, the transcription from *otrAp1* shuts down, and *otcCp1* starts to lead a polycistronic transcript from two upstream structural tailoring genes, *otcZ* (methyltransferase) and *otcC* (hydroxylase). Therefore *otrA* is always expressed. Before the onset of antibiotic biosynthesis, transcription from *otrAp1* protects bacteria from neighbouring cells that make oxytetracycline. When endogenous biosynthesis begins, the continued expression of resistance is ensured and cooperates with synthesis.
Figure 1.2 Oxytetracycline/tetracycline

(A) Structures of tetracycline family antibiotics. (B) Resistance mechanism of Ribosomal Protection Protein: Blockage at A site by tetracycline is cleared by RPP so that the EF-Tu+aa-tRNA+GTP complex can make access to the ribosome and continue with peptide binding and translocation. The elongation is returned to normal. (C) Oxytetracycline biosynthetic gene cluster in S. rimosus. Structural genes are in blue, regulatory gene in green and resistance gene in red. Two promoters (otrAp1 and otcCp1) direct otrA (RPP gene) transcription at exponential growth stage and stationary growth stages when bacteria start to synthesize the oxytetracycline, respectively.
1.3.2 Novobiocin

Novobiocin is an aminocoumarin antibiotic produced by *Streptomyces sphaeroides*. Other classical aminocoumarin compounds include clorobiocin (from *Streptomyces roseochromogenes*) and coumermycin A1 (from *Streptomyces rishiriensis*). All three contain a substituted aminocoumarin core moiety: a deoxysugar is attached to the C7 (Figure 1.3A). Structural similarity and distinction of these compounds are reflected in their biosynthetic gene clusters, which are unidirectionally arranged with similar organization (Figure 1.3B). In addition, *nov/clo/couEG* reside on the 5’ end of the cluster and encode positive regulators of aminocoumarin biosynthesis. All three clusters include self-resistance genes at the 3’ end: an aminocoumarin resistant gyrase B subunit (*gyrBR*) for all three; while clorobiocin and coumermycin A1 clusters have an additional gene *parYR*, encoding a resistant subunit of topoisomerase IV. These resistance genes protect against the aminocoumarin effect on DNA replication.

In bacterial cells, replication and transcription strongly influence DNA superhelical density. Ahead of the replication fork or transcription apparatus, DNA gets overwound and gains positive supercoiling, while behind the site of replication or transcription, the DNA is negatively supercoiled. Maintenance of the proper topological state of DNA is controlled by complementary topoisomerases. Type II topoisomerase (e.g. DNA gyrase) introduces negative supercoils or relieves positive ones ahead of the replication fork/transcription complex, while Type I topoisomerase reduces negative supercoils behind replication/transcription (though it has no effect on positive supercoils).
Aminocoumarins apply their therapeutic activity by binding tightly with the B subunit of DNA gyrase, interrupting the maintenance of the essential DNA topological balance by this enzyme\(^\text{31}\). Aminocoumarin producers thus harbor isoforms of gyrase B subunits that are resistant to these endogenous antibiotics as the backup to the constitutively expressed sensitive subunits\(^\text{31}\). Since the promoters that direct topoisomerase gene expression are also vulnerable to aminocoumarins, the resistant DNA gyrase (e.g. \(\text{gyrB}^R\)) needs to be expressed prior to the appearance of antibiotics. A transcriptional connection between aminocoumarin biosynthesis and self-resistance was recently studied (Figure 1.3C)\(^\text{32}\).

Eight DNA regions with promoter activities were revealed in the novobiocin biosynthetic gene cluster. Further comparison between transcripts from intact and disrupted novobiocin clusters uncovered a single operon from the regulatory gene \(\text{novG}\) to the structural gene \(\text{novW}\) with three internal promoters of minor significance\(^\text{32}\). Expression of these genes started at least 48 hours before the onset of novobiocin production and decreased after peak value but was maintained at a detectable level\(^\text{32}\). More interestingly, the initial peak of \(\text{gyrB}^R\) expression was found to be synchronous with that of upstream structural genes (e.g. \(\text{novH}\) and \(\text{novOPQ}\)), whereas a second peak of expression was found when novobiocin concentrations started to increase, which agreed with early observations that activity of the \(\text{gyrB}^R\) promoter was induced when cultivated with novobiocin\(^\text{32}\). It has been suggested that \(\text{gyrB}^R\) is co-transcribed early with the upstream structural genes. Once novobiocin accumulates in the cell, a second round of transcription is initiated by its own promoter as a strengthened response to the gyrase inhibitor\(^\text{32}\).
Figure 1.3 Novobiocin

(A) Structure of aminocoumarins: novobiocin, clorobiocin and coumermycin A. (B) Biosynthetic gene clusters of all three aminocoumarins. Structural genes are coded in blue, regulatory genes in green and resistance genes in red. The turning arrows indicate confirmed promoters. (C) Transcription of resistance gene $gyrB^R$ is coordinated with novobiocin biosynthesis: its early expression is along with upstream structural genes which are likely directed by $novGp$. Once the novobiocin starts to accumulate within the cell culture, a strengthened expression is led by its own promoter, $gyrB^Rp$.

1.3.3 Spiramycin

Spiramycin is a macrolide antibiotic that inhibits protein synthesis. It is composed of a 16 membered polyketide macrocyclic lactone (platenolide) attached to three deoxysugars (mycaminose, forosamine and mycarose) (Figure 1.4A)\textsuperscript{33}. The primary self-
resistance exhibited by its producer, *Streptomyces ambofaciens*, is by post-translational methylation of its own 23S ribosomal RNA, though other types of resistance such as drug-inactivation by esterase or kinase, as well as ATP-dependant efflux are also employed.\(^4\)

The biosynthetic gene cluster of spiramycin spans 85 kb of continuous DNA and consists of 44 genes (Figure 1.4B)\(^33\). Five type I polyketide synthase genes (*srmGI* to *srmGV*, occupy about 40 kb) for platenolides sit in the middle, flanked with genes encoding enzymes for sugar attachments\(^33\). Three putative resistance genes were found within the cluster\(^33, 35, 36\): (1) *srmB*, encoding an ATP binding component of an ABC transporter; (2) *srm29*, encoding a glycosidase that may reactivate glycosylated and inactive spiramycin (macrolide inactivation/reactivation will be discussed later in oleandomycin producer *S. antibioticus*); and (3) *srmD*, encoding an ErmE-like methyltransferase that modifies the 23S rRNA\(^37\). Although *srmB*, *srm29* and *srmD* are all transcribed as individual operons under respective promoters, the onset of transcription for two of them, *srmB* and *srm29*, might be controlled by the same pathway-specific regulators Srm28c along with most biosynthetic genes in the cluster. Srm28c is activated by SrmR (Figure 1-4C)\(^35\). This suggests that expressions of spiramycin efflux pump and enzymes for inactivation/reactivation of the drug are initiated concurrently with spiramycin biosynthesis.
Figure 1. 4 Spiramycin

(A) Structure of spiramycin. (B) Biosynthetic gene cluster of spiramycin: structural genes in blue, regulatory genes in green, resistance genes in red and uncharacterized gene in pink. Other than srmR and srm28c described in the text, the third regulatory gene, srm13c, encodes a putative GTPase which may act as a molecular switch that mediates various cellular processes. Its expression was always abundantly detected and independent of the other two regulators and genes under its control are not clear. Three resistance strategies are involved: (1) the srmD encoded methyltransferase modifies 23S rRNA. In addition, (2) gimA and gimB outside the gene cluster are bracketed and encode a glycosylase that temporarily modify and inactivate nascent spiramycin which gets reactivated by srm29 encoded glycosidase after it is pumped outside the cell by (3) srmB encoded ABC transporter. (C) Activations of srmB and srm29, along with spiramycin biosynthetic genes are controlled by the pathway specific regulators SrmR and Srm28c.
1.4 Resistance involved in prodrug processing or drug sequestration

1.4.1 Nisin

Nisin is the best characterized lantibiotic (Figure 1.5A) that is often used as an additive to dairy products or canned food to prevent the growth of Gram-positive bacteria, especially *Clostridia, Listeria, Bacilli, and Staphylococci*. This ribosomally synthesized amphiphilic cationic peptide interrupts the bacterial membrane potential, forms voltage-dependant pores and causes an efflux of ions and low molecular weight molecules driven by proton gradient. The membrane of nisin producing bacteria, *Lactococcus lactis*, is also sensitive to this action and the bacterium has developed a very complex mechanism to protect itself. Early primer extension analysis along the 15 kb nisin biosynthetic clusters revealed three transcripts corresponding to early and late stages of nisin biosynthesis and resistance (Figure 1.5B): *nisABTCIP, nisRK* and *nisFEG*. Nisin precursors are subsequently synthesized and modified by NisA/NisB/NisC in the cytoplasm and the prodrug of nisin (Nisin C) is translocated to the outside of the membrane by NisT where the leader sequence is cleaved by the protease NisP to produce the mature product. The nascent nisin is captured by a sequestering protein NisI and is also sensed by a two-component regulation system of NisK (sensor kinase) and NisR (response regulator). Finally, NisR activates ABC transporter NisEFG to pump out the drug.

The whole nisin resistance system consists of two steps: co-transcription of *nisT* and *nisI* with *nisA, nisB, nisC and nisP* ensures coherence of biosynthesis, translocation,
processing and sequestering, which buffers the attack from the antibiotic and gains time for NisKR to sense the molecule and activate NisFEG transporter.

Figure 1. A) Structure of nisin. (B) Biosynthetic gene cluster of nisin: structural genes in blue, regulatory genes in green and resistance genes in red. The turning arrows indicate confirmed promoters. A putative promoter located before nisI (indicated by the smaller arrow) was reported. (C) A comprehensive resistance system to nisin involved early translocation of precursors, sequestering of mature products and late induction of ABC transporter. Translocase NisT exports pro-drug NisC, which is processed by NisP to form mature product Nisin. nisl, which is likely co-transcribed with the structural genes nisA, nisB and nisC, encodes the sequestering protein that catches the nascent nisin. Finally, nisin itself is the signalling molecule that activates the ABC transporter NisFEG via the NisK-NisR two-component system.
1.4.2 Oleandomycin

Belonging to the macrolide antibiotics, oleandomycin has a very similar structure (Figure 1.6A) to other members of this family (e.g. erythromycin) and its target site is also located within the ribosome. However, unlike some macrolide producers that possess specific methyltransferases that mono/dimethylates their own ribosomal RNAs, the oleandomycin producer, *Streptomyces antibioticus* expresses sensitive ribosomes through the whole cell life cycle, even during antibiotic biosynthesis. Instead, this organism employs a different resistance mechanism. A gene (*oleD*) encoding a glycosyltransferase was first isolated and characterized outside the oleandomycin biosynthetic gene cluster of *S. antibioticus* and was found to be highly similar to macrolide glycosyltransferase from at least 15 other polyketide producing *Streptomyces* spp. Later on, a second gene (*oleI*) encoding a glycosyltransferase that showed more specificity and stronger glycosylation activity to oleandomycin was identified within the oleandomycin gene cluster (Figure 1.6B). In the vicinity of *oleI*, another gene (*oleR*) encoding a glycosidase was found. Two additional oleandomycin resistance genes (*oleB* and *oleC*) have been identified that encode ABC transporters for oleandomycin. *oleB* is located at the 3’ end of the oleandomycin gene cluster and its product has been shown to undergo conformational changes via interaction with glycosylated oleandomycin, leading to secretion of this prodrug across the membrane. Taking the roles played by OleI (glycosyltransferase), OleR (glycosidase) and OleB/OleC (ABC transporter) together, Mendez and Salas group proposed a model for oleandomycin self-resistance that tightly
couples the oleandomycin biosynthesis with its immunity (Figure 1.6C): First, in the presence of UDP-glucose, OleI prevents appearance of intracellular active compounds by glycosylating the free 2’-OH of the desosamine sugar moiety of either the final product or some late oleandomycin biosynthetic intermediate. It is possible that oleandomycin biosynthetic enzymes are associated in a complex that channels intermediates from one enzymatic unit to another. Glycosyltransferase OleI may be involved in the last few biosynthetic steps and converts the final product to the inactive glycosylated prodrug. Then, the prodrug is exported by OleB and/or OleC and regains its activity outside the membrane where OleR removes the additional glycosyl group.
Figure 1.6 Oleandomycin

(A) Structure of oleandomycin. (B) Biosynthetic gene cluster of oleandomycin: structural genes in blue, and resistance genes in red. \textit{oleD} and \textit{oleC} outside the gene cluster are in brackets and encode a less specific glycosyltransferase and a transporter respectively. (C) A complex resistance system of oleandomycin involved glycosylation of oleandomycin or its intermediates from the pathway by OleI, translocation of the inactivated drug by OleB/C and reactivation of the drug outside the cell by OleR. The late tailoring enzymes and resistance proteins might form a complex to process the intermediates/oleandomycin more efficiently.
1.4.3 Streptomycin

Streptomycin is an aminoglycoside antibiotic that consists of aminocyclitol (streptidine), 6-deoxyhexose (streptose), and $N$-methyl-L-glucosamine moieties formed by independent biosynthetic pathways\textsuperscript{47, 48}. It inhibits protein synthesis by binding to the 16S rRNA of the 30S subunit of the bacterial ribosome\textsuperscript{47, 48}. A similar inactivation-secretion-reactivation strategy is taken by streptomycin producer, \textit{Streptomyces griseus} as seen for oleandomycin resistance (Figure 1.7A): The major resistance gene, \textit{aphD} (or \textit{strA}), encoding a streptomycin 6-phosphotransferase, was found within the streptomycin gene cluster and was surrounded by biosynthetic and regulatory genes (Figure 1.7B)\textsuperscript{47, 48}. AphD is likely involved in phosphorylation of streptidine moiety of the streptomycin\textsuperscript{47, 48}. Some early studies showed that this phosphorylation might occur during the biosynthesis of the amidinostreptamine (the precursor of streptidine) though it is not clear whether this 6-phosphate is transferred by \textit{aphD} encoded phosphotransferase and carried through the whole streptomycin biosynthesis\textsuperscript{49-51}. If this is true, phosphorylation on the intermediate might not only be an early preparation of resistance but also play as a key step for the rest of the biosynthesis (Figure 1.7C): the streptidine-6-phosphate would link streptose to form a disaccharide precursor. 2-OH of this disaccharide displaces the NDP of NDP-$N$-methyl-L-glucosamine to generate the inactive precursor dihydrostreptomycin-6-phosphate\textsuperscript{47, 48}. The inactive precursor is specifically exported by the ABC transporter encoded by \textit{strV} and \textit{strW}\textsuperscript{47, 48}. During this transmembrane passage, the precursor is oxidized from CH$_2$OH state to CHO state, giving streptomycin-6-phosphate\textsuperscript{47, 48}. Then, the
6-phosphate group is removed in the extracellular space by a secreted phosphatase encoded by \textit{strK}, generating an active antibiotic\textsuperscript{47,48}.

Furthermore, expression of \textit{aphD} also coordinates with biosynthetic genes (Figure 1.7B)\textsuperscript{47,52}: Transcription of \textit{aphD} is directed by two promoters. Before the antibiotic biosynthesis takes place, \textit{aphDp1} is turned on constitutively, directing cotranscription of \textit{aphD} with the upstream pathway specific activator \textit{strR}\textsuperscript{47,52}. The second promoter \textit{aphDp2} is located within \textit{strR} gene and is activated when \textit{S. griseus} starts to synthesize streptomycin, probably by the StrR regulator that positively controls expression of almost all genes in the cluster\textsuperscript{52}. Therefore, expression of \textit{aphD} follows the action of the pathway specific regulator which runs from the early to late phase of streptomycin production and guarantees a prompt provision of the drug’s inactivating enzyme.
Figure 1.7 Streptomycin

(A) Structure of streptomycin. (B) Biosynthetic gene cluster of streptomycin: structural genes in blue, regulatory genes in green and resistance genes in red. Turning arrows indicate confirmed promoters. The black boxes indicate StrR binding sites. The resistance gene $\textit{aphD}$ is co-transcribed with $\textit{strR}$ at early stage and later turns on its own promoter which is positively regulated by StrR along with other biosynthetic genes. (C) A comprehensive resistance system of streptomycin. 1-D-Glucose was processed in three branched pathways and gives rise to three saccharide intermediates (Streptidine, dihydrostreptose and methyl-glucosamine). Streptidine is phosphorylated to form...
streptidine-6P which might be catalyzed by a phosphotransferase (AphD?). This phosphorylated intermediate is linked with other two saccharide intermediates to form dihydrostreptomycin-6-P. Oxidation on the pentose moiety of dihydrostreptomycin-6-P is completed during transportation by StrVW. Dephosphorylation is then achieved by StrK outside the membrane.

1.4.4 Fosfomycin

The presence and characterization of natural compounds containing a unique carbon-phosphorus bond in their structure are quite limited, but most of them show important bioactivities. Fosfomycin is a well-studied example of this group of antibiotics and possesses an epoxide (Figure 1.8A)\(^{53}\). It is produced by a variety of microorganisms, including *Streptomyces wedmorensis, Pseudomonas syringae* and *Pseudomonas viridiflava*\(^{53}\). As an analogue of phosphoenoyloyruvate (PEP), this antibiotic inhibits PEP UDP-N-acetylglucosamine-3-O-enoylpyruvyltransferase (MurA) in the first step of peptidoglycan biosynthesis\(^{53}\). Since fosfomycin does not inhibit other enzymes using PEP, it provides great specificity against bacteria but shows almost no toxicity to humans\(^{53}\).

In *S. wedmorensis*, fosfomycin is synthesized in four steps and the first two are common in all producing bacteria of compound containing carbon-phosphorus bond (Figure 1.8C)\(^{54}\). The first step is the isomerization of PEP by PEP phosphomutase to produce phosphonopyruvic acid (PnPy); the second step is the decarboxylation of PnPy to generate phosphonoacetaldehyde (PnAA); then the PnAA is methylated to provide 2-hydroxypropylphosphonic acid (HPP), which is converted to the final product via epoxide formation in the fourth step. Genes (*fom1* to *fom4*) responsible for these four biosynthetic steps have been identified from the fosfomycin gene cluster in *S. wedmorensis* (Figure 1.8B)\(^{54}\). In addition, six other genes were found within the same cluster and named *fomA*.
to \textit{fomF} \cite{54}. Products of \textit{fomC} and \textit{fomF} show weak similarity to the yeast alcohol dehydrogenase and a putative amino acid racemase respectively, whereas the functions of \textit{fomD} and \textit{fomE} are still unclear \cite{54}. However, combined expression of \textit{fomA} and \textit{fomB}, or individual expression of \textit{fomA} but not \textit{fomB} in \textit{E. coli} conferred very high resistance to fosfomycin (MIC $>$800 µg/ml) \cite{55,56}. In addition, fosfomycin could be converted to inactive forms (fosfomycin mono/diphosphate) in the presence of ATP by crude extracts from these transformed \textit{E. coli} cells and prolonged incubation caused the reappearance of active fosfomycin \cite{55}. These observations suggested fosfomycin resistance mechanism was by reversible phosphorylation of the fosfomycin molecule. Further structural analysis determined that gene products of \textit{fomA} and \textit{fomB} encoded respectively a fosfomycin monophosphatase and a fosfomycin diphosphatase \cite{57}.

Two questions are remaining: (1) How does \textit{S. wedmorensis} reactivate its phosphorylated fosfomycin and transport it across the cell membrane? (2) Since the resistance is actually conferred by monophosphorylation catalyzed by FomA, why there is a need for a second phosphate group added by FomB? Researchers have proposed a model for fosfomycin export/reactivation (Figure 1.8C) \cite{55,56}: fosfomycin synthesized in the cytoplasm is immediately converted to its inactive monophosphorylated form by FomA for self-resistance. Then, fosfomycin monophosphate is phosphorylated by FomB to generate fosfomycin diphosphate. Now the compound possessing tri-phosphate group resembles the structure of ATP and could be used as energy supply for excretion. After liberation of free energy stored in the diphosphate bond, the compound returns to its
active form and is pumped across the membrane. This hypothesis is quite novel and attractive as it suggested not only the cooperation between biosynthesis and resistance, but also a tight connection between two resistance determinants (drug inactivation and efflux). However, the phosphatase and exporter involved in hydrolysis of diphosphate bond and excretion are still unknown.

**Figure 1.8 Fosfomycin**

(A) Structure of fosfomycin. (B) Biosynthetic gene cluster of fosfomycin. *fomA* and *fomB* in red encode enzymes phosphorylate fosfomycin intermediates. (C) Biosynthesis of fosfomycin is processed by enzymes encoded by *fom1*, *fom2*, *fom3* and *fom4*, which is immediately followed by two steps of phosphorylation that temporarily inactivate the antibiotic and perhaps provide driving forces for transport at mean time.

1.5 Resistance triggered by bioactive molecules from biosynthetic pathway

The above examples all suggest that the expression and functional involvement of resistance proteins tightly couples the biosynthesis of their relevant bio-active molecules. On the other hand, although not all these examples provided direct evidence, it is
reasonable to hypothesize that these products could be triggering molecules to initiate
immunities. Signals could be sensed by and passed to regulators that mediate downstream
resistance and/or biosynthetic genes, like the aforementioned two-component regulatory
system NisRK that responds to nisin and turns on expressing of the ABC transporter
NisEFG (Figure 1.5).

Another well-known model of this antibiotic dependant regulation on resistance
genes was first observed in tetracycline resistant E. coli, in which a tetA encoded efflux
pump/resistance determinant was found on a transposon Tn10. Today, over 20
different types of tetracycline pumps have been identified, mostly encoded by mobile
genetic element such as transposon, plasmid and integron in pathogenic bacteria, although
rare chromosomal encoded cases were reported in Staphylococcus aureus and
Helicobacter pylori. Expression of tet genes are inducible by tetracycline in both Gram-
positive and Gram-negative pathogens though the induction mechanisms are different.
For the former, tet genes are mostly controlled by a “translational attenuation”
mechanism, while the latter are mediated by tetracycline responsive repressor TetR. In a
conventional tetA-tetR model (Figure 1.9), the repressor protein TetR binds to the
palindromic operator sites within the intergenic region between tetA and tetR, hampers
promoter activities of both genes and turns off tetA expression. When tetracycline
enters the cell, these antibiotic molecules bind TetR and change its conformation, leading
to the release of repressor proteins from the promoter region and the expression of tetA.
Although efflux pumps were recognized as a type of resistant determinant first in pathogenic bacteria, it has been suggested that their origins, especially the drug-specific ones, are from drug-producing microorganisms such as *Streptomyces* \(^{60,62}\). In fact, efflux genes are commonly seen within drug biosynthetic clusters, and it is not rare to see that genes for efflux pumps are paired with adjacent regulatory genes (e.g. *rifP-rifQ* for rifamycin \(^{63}\), *slgT-slgR1* for streptolydigin \(^{64}\) and *kirT-kirR* for kirromycin \(^{65}\).

**Figure 1.9** Tetracyling mediated TetR-TetA resistance

tetR and tetA are divergently transcribed. In the absence of tetracycline, TetR proteins bind operator sequences within intergenetic regions between tetR and tetA and shut off the transcription of both genes. A conformational change of TetR is induced when binding to tetracycline and leads to the release of TetR from DNA. TetA exporter is then expressed and tetracyclines are pumped out.

Other than cases where resistant apparatus are “obligated” to recognize drug intermediate or pro-drug as “ligands” and turned on before the final product is completed (e.g. OleI-OleB/C-OleR in oleandomycin biosynthesis/export (Figure 1.6) and AphD-StrVW-StrK in streptomycin biosynthesis/export (Figure 1.7)), only a few characterized resistance determinants, including efflux pumps, provided evidence of direct activation by
endogenous bioactive molecules via an antibiotic responsible regulator. But more
importantly, some examples also suggested that antibiotic intermediates from biosynthetic
pathway might act as early signals and pave the way forward in their biosynthetic
pathway, establishing resistance early during biosynthesis, which we proposed as the
“feed-forward” mechanism\(^66\).

1.5.1 Simocyclinone

Simocyclinone D8 is a newly identified member of the aminocoumarin family of
antibiotics produced by \textit{Streptomyces antibioticus} Tü 6040\(^67\). Unlike the other three
compounds of this family (novobiocin, clorobiocin and coumermycin, Figure 1.3),
simocyclinone inhibits DNA gyrase in a novel mode of action by binding to the A subunit,
though a new binding site with B subunit was recently reported\(^67\text{-}69\).

Structurally, simocyclinone D8 is a hybrid molecule of a coumarin moiety and an
angucyclinone polycyclic ring system, and a linear octatetraene dicarboxylic acid
connected to a deoxysugar bridges the two together (Figure 1.10A)\(^67\). The simocyclinone
biosynthetic gene cluster spans 39.4 kb region and consists of 38 ORFs (Figure 1.10B)\(^67\).

Striking sequence similarities were found for six coumarin biosynthetic genes and 12
angucyclinone biosynthetic genes to other antibiotics with the same structural moieties\(^67\).
However, instead of genes coding for a resistant gyrase subunit, a divergently transcribed
gene pair, \textit{simX} (also known as \textit{sim17}) and \textit{simR} (also known as \textit{sim16}) that resembles
\textit{tetA-tetR} pair of tetracycline resistance localize within the biosynthetic gene cluster
(Figure 1.10 B and C)\(^67,70\). It was found that expression of \textit{simX}, a putative exporter gene,
under a strong heterologous promoter in *Streptomyces lividans* conferred high resistance of simocyclinone D8 to this sensitive strain, indicating it is a real resistance determinant. Two distinct operator sites were also identified within the *simX-simR* intergenic region and DNA binding by SimR could be completely abolished by simocyclinone D8 at a concentration of 62.5 μM in a gel mobility shift assay. Furthermore, promoters of *simX* and *simR* could both be induced by exogenously added simocyclinone D8 in the presence of SimR *in vivo*, suggesting a connection of efflux/resistance to the biosynthesis of the end product. More interestingly, one natural intermediate, simocyclinone C4, which lacks the coumarin moiety and thus has no antibiotic activity, was found to dissociate SimR from DNA binding at higher concentration (250 μM) and activate promoters of *simX* and *simR in vivo* by luminescent assays.
Figure 1. 10 Simocyclinone D8

(A) Structure of simocyclinone D8. (B) Biosynthetic gene cluster of simocyclinone D8. Structural genes are in blue, regulatory genes are in green and resistance genes are in red. (C) A TetR-TetA like export and resistance of simocyclinone D8 via SimR-SimX. The late intermediate simocyclinone C4 can bind SimR as well and release it from operators. However, induction of simX expression by this intermediate is less efficient than that by the final product, simocyclinone D8.

1.5.2 Landomycin

Produced by *Streptomyces cyanogenus*, the landomycin family of antibiotics includes a benzoanthraquinone type aglycone moiety attached by a varying lengths of carbohydrate chain that determines the bioactive potency to inhibit DNA synthesis (Figure 1.11A). Landomycin A is the final product that possesses the longest glycoside chain (hexasaccharide) and is the most active landomycin. Its gene cluster covers an
approximately 35 kb DNA region and consists of 32 ORFs (Figure 1.11B), including three genes encoding type II iterative polyketide synthase. The only putative self-resistance gene within the cluster is \textit{lanJ} which encodes a landomycin efflux pump and is cotranscribed with several downstream genes involved in late conversion of landomycin D (a disaccharide precursor) to landomycin A (Figure 1.11C). Divergently located upstream of \textit{lanJ}, is the regulatory gene encoding a TetR-like repressor, \textit{lanK}. Like TetR, LanK represses expression of \textit{lanJ}, which can be relieved by either landomycin A or two intermediates with shorter glycoside chain, landomycin E (trisaccharide) and landomycin B (pentasaccharide) (Figure 1.11C). As \textit{lanJ} is transcriptionally linked with genes involved in late biosynthetic steps, assembly of late biosynthetic enzymes cannot be initiated until the exporter gene is activated. Therefore, landomycin precursors are likely the primary inducers to turn on expression of both exporter and late biosynthetic enzymes. This basal transcription from \textit{lanJ} promoter might be further strengthened by a regulator encoded by \textit{lanI} at the end of the landomycin gene cluster. Finally, LanJ exports molecules with longer sugar chain and stronger bioactivity.
Figure 1. 11 Landomycin

(A) Structure of landomycin. (B) Biosynthetic gene cluster of landomycin. Structural genes are in blue, regulatory genes are in green and resistance genes are in red. The turning arrow indicates a long transcript directed by lanJ promoter and going through late biosynthetic genes from lanZ1 to lanGT4. (C) LanK-LanJ mediated TetR-TetA like landomycin export and resistance. In addition to the mature landomycinA, intermediates with less antibiotic potency are primary inducers that lead to basic level expressions of lanJ and late biosynthetic genes in the same operon.
1.5.3 Doxorubicin

Doxorubicin and its pathway precursor daunorubicin are clinically important antitumor agents produced by *Streptomyces peucetius*. Like tetracycline, they are also type II polyketides using propionyl-CoA as the starter unit and malonyl-CoA as extenders to build up carbon backbone of the 12-deoxyalkanonic acid (Figure 1.12A). This condensation process is carried out by the polyketide synthase (PKS) complex encoded by *dpsABCDEFGY*, which are clustered with other tailoring genes that lead to daunorubicin and the end product doxorubicin (1.12B).

At least three resistance genes were found in the biosynthetic gene cluster, *drrA* and *drrB* that together form ATP-dependant efflux pumps, while *drrC* mediates a novel type of resistance. Studies by the Huchinson group showed that, even in the presence of *drrAB*, *drrC* mutants can be only isolated from a doxorubicin non-producing background and exhibited increased drug sensitivity, suggesting that *drrC* is essential for antibiotic biosynthesis and self-resistance. The DrrC protein shows sequence similarity to the *E. coli* UvrA protein, a DNA binding protein involved in excision repair during DNA damage. This damage is caused by free radicals produced from interaction between O₂ and reduced DNA-bound daunorubicin or doxorubicin. Therefore, DrrC protein was speculated to shield genomic DNA from drug binding in a fashion similar to UvrA, although the expression of DrrC in a *E. coli* UNC523 *uvrA* mutant did not fully complement the lethal effect from UV, a conventional method that causes excision damage in DNA.
Expression of \textit{drrC}, as well as two other resistance genes \textit{drrA} and \textit{drrB}, is under the control of DnrI, the pathway specific activator of doxorubicin biosynthesis\textsuperscript{81}. DnrI is homologous to other pathway specific regulator, such as ActII-orf4 from actinorhodin biosynthetic pathway in \textit{S. coelicolor}, and belongs to the SARP (\textit{Streptomyces} antibiotic regulatory protein) family\textsuperscript{81}. In addition, DnrI is regulated by another regulatory protein encoded by \textit{dnrN}\textsuperscript{82}. Transcriptional analysis showed that DnrI binds directly to the promoter region of structural genes that are involved in early steps of doxorubicin biosynthesis (Figure 1.12B)\textsuperscript{82}.

Furthermore, inactivation of \textit{dnrI} and \textit{dnrN} not only abolished the production of the final product and some intermediate (e.g. \(\varepsilon\)-Rhodomycinone, transformed from upstream intermediate Alkavinone by DnrF), but also inhibited transcription of biosynthetic genes (e.g. \textit{dnmLM}, \textit{dnrXY} and \textit{dnmZUV}) and resistance genes (\textit{drrAB} and \textit{drrC})\textsuperscript{81-83}. These findings suggested DnrI is the actual regulator to most if not all of the biosynthetic and resistance genes and DnrN exerts its influence via DnrI indirectly.

More interestingly, a third regulatory gene, \textit{dnrO}, is divergently located downstream of \textit{dnrN} (Figure 1.12B). DnrO binds specifically to the \textit{dnrN-dnrO} intergenic region, positively regulates \textit{dnrN} transcription but negatively mediates its own expression\textsuperscript{83}. Autorepression by DnrO can be relieved in the presence of molecules from intermediate rhodomycin D to final product doxorubicin, indicating an interaction between them (Figure 1.12C)\textsuperscript{83}. An association between resistance and biosynthesis via pathway specific regulation was therefore suggested (Figure 1.12C)\textsuperscript{83}: DnrO might have two binding sites
within the \textit{dnrO-dnrN} intergenic region. Binding to \textit{dnrO} proximal site represses the \textit{dnrO} expression, while binding to \textit{dnrN} proximal one activates \textit{dnrN} expression. Interaction with rhodomycin D and other intermediates can prevent DnrO binding to the former site but not the latter. Therefore, once the first glycosylated intermediate rhodomycin D is synthesized, derepression of DnrO is triggered and the increased level of DnrO enhances \textit{dnrN} expression which then fully activates \textit{dnrI}, leading to the expression of resistance and late biosynthetic genes.
Figure 1. 12 Doxorubicin

(A) Structures of doxorubicin and daunorubicin. (B) Biosynthetic gene cluster of doxorubicin. Structural genes are in blue, regulatory genes are in green and resistance genes are in red. The turning arrows indicate confirmed promoters, most of which are controlled by the pathway specific activator DnrI. Though annotated as a resistance gene, function of drrD is not clear. (C) A brief doxorubicin biosynthetic pathway and a cascade of regulation on doxorubicin export/resistance. Late intermediates such as rhodomyccin D as well as daunorubicin and doxorubicin can interact with and probably release the autorepression of dnrO. DnrO activates expression of dnrN, and DnrN in turn activates dnrI. The pathway specific activator DnrI initiates genes for doxorubicin biosynthesis and resistance.
1.6 Objectives of this work

The examples I have discussed above, suggest that there is tight coordination between antibiotic biosynthesis and antibiotic resistance in antibiotic producers. Some examples also suggest the involvement of biosynthetic intermediates in the activation of resistance genes (e.g. landomycin resistance), or a cascade of regulation was triggered by intermediates that lead to late biosynthetic steps and expression of resistance genes (e.g. doxorubicin resistance). However, at the outset of my work, it was unknown whether these intermediates indeed accumulate in the cytoplasm and serve as intracellular signals to initiate resistance/export. Furthermore, how the resistance genes are activated and coordinated across the entire bacterial community was also unknown: this is important because streptomycetes and other antibiotic producing bacteria are known to generate specialized cell types not all of which produce the biologically active secondary metabolites. Therefore, my PhD thesis project was to investigate the intracellular and intercellular signalling processes that coordinate the resistance with biosynthesis of bioactive molecules in producing bacteria. The focus of this work was to build up a model of how actinorhodin biosynthesis and resistance/export are coordinated in S. coelicolor. Once detailed understanding is obtained, we can apply the knowledge and tools to other bioactive molecules produced by S. coelicolor or other Streptomyces strains. This work would also provide clues of the action mode or the target of actionrhodin.
Table 1. Examples of resistance and biosynthesis coordination in producing bacteria of bioactive molecules

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>producing bacteria</th>
<th>Target or action mode</th>
<th>Resistance genes</th>
<th>Resistance strategies</th>
<th>Coordination with biosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pantolentolactone</td>
<td><em>Streptomyces avermitilis</em></td>
<td>Attacking glyceraldehyde-3-phosphate dehydrogenase (GAPDH).</td>
<td><em>gap1</em></td>
<td>Synthesis of resistant GAPDH</td>
<td>Unknown</td>
</tr>
<tr>
<td>Tetracycline (tetracycline, oxytetracycline, chlortetracycline)</td>
<td><em>Streptomyces spp.</em> (S. aureofaciens, S. rimosus, and S. viridofaciens) and some gram-negative bacteria</td>
<td>Inhibiting protein synthesis by blocking the attachment of charged aminoacyl-tRNA to the A site on the 30S subunit ribosome. (1)</td>
<td><em>tetA (otrB)</em> and homologue s, <em>tet(O) (otrA)</em> and homologue s</td>
<td>Efflux pumps, Ribosome Protection Protein (RPP)</td>
<td>Tetracycline triggering export, cotranscription with biosynthetic genes</td>
</tr>
<tr>
<td>Aminoglycoside (kanamycin, gentamicin, sisomicin, tobramycin)</td>
<td><em>Streptomyces spp.</em> (S. kanamyceticus and S. tenebrarius) and <em>Micromonospora spp.</em> (M. rosea, M. zionensis and M. purpurea)</td>
<td>Inhibiting protein synthesis by binding 30S subunit of ribosome and blocking translocation of peptidyl-tRNA from A to P site. (2)</td>
<td><em>kmr</em> homologue s</td>
<td>Methylation of 16S rRNA in 30S subunit</td>
<td>Unknown</td>
</tr>
<tr>
<td>Lincomycin</td>
<td><em>Streptomyces lincolnensis</em></td>
<td>Binding 50S subunit of ribosome and Inhibiting protein synthesis by preventing peptide bonding, aminoacyl translocation and causing premature dissociation of the peptidyl-tRNA from the</td>
<td><em>lmrA, lmrB, lmrC</em></td>
<td>Efflux pumps, Methylation of 23S rRNA of 50S subunit</td>
<td>Unknown</td>
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<tr>
<td>Antibiotic</td>
<td>Producing Organism</td>
<td>Mode of Action</td>
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<tr>
<td>Aminocoumarin (novobiocin, clorobiocin, coumermycin)</td>
<td><em>Streptomyces spp.</em> (<em>S. sphaeroides</em>, <em>S. roseochromogenes</em> and <em>S. rishiriensis</em>)</td>
<td>Binding to B subunit of gyrase and inhibiting the ATP-dependent DNA supercoiling catalysed by gyrase.</td>
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<td>Synthesis of resistant gyrase B subunit or topoisomerase IV</td>
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<td>Cotranscription with biosynthetic genes</td>
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<tr>
<td>Erythromycin</td>
<td><em>Saccharopolyspora erythraea</em></td>
<td>Binding to 50S subunit of ribosome and interfering aminoaeryl translocation.</td>
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<td></td>
<td><em>ermE</em> Methylation of 23S rRNA in 50S subunit</td>
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<td></td>
<td>Expression in accordance with activation of biosynthetic genes</td>
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<tr>
<td>Spiramycin</td>
<td><em>Streptomyces ambofaciens</em></td>
<td>Binding to 50S subunit of ribosome and interfering aminoaeryl translocation.</td>
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<td><em>ginA and gimB</em> (outside the spiramycin gene cluster), <em>srmB</em>, <em>srm29</em> and <em>srmD</em></td>
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<td>Efflux pumps, glycosylation of spiramycin and methylation of 23S rRNA of 50S subunit</td>
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<td>Regulation by SrmR and Srm28c</td>
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<tr>
<td>Nisin</td>
<td><em>Lactococcus lactis</em></td>
<td>Breaking down cell membrane potential and causing leakage of ions and small molecules.</td>
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<td>nisT, nisl, nisF, nisE and nisG Efflux pumps and sequestering protein</td>
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<td>Cotranscription with biosynthetic genes and nisin triggering export via NisK-NisR</td>
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<tr>
<td>Mitomycin</td>
<td><em>Streptomyces lavendulae</em></td>
<td>Cross linking DNA and blocking DNA replication.</td>
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<td></td>
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<td>mcrA (outside the mitomycin gene cluster) and homologues (mitR and mmcM), mrd, met</td>
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<td>Mitomycin oxidase, sequestering protein and efflux pumps</td>
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<td>Cotranscription with biosynthetic genes or mitomycin triggering export via MitQ?</td>
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<tr>
<td>Oleandomycin</td>
<td><em>Streptomyces antibioticus</em></td>
<td>Binding to 50S subunit of ribosome and interfering aminoaeryl translocation.</td>
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<td>oleI, oleR, oleB/oleC Oleandomycin glycosylation and efflux pumps</td>
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<td></td>
<td>Functional involvement of exporter and glycosyltransferase in biosynthesis</td>
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<tr>
<td>Antibiotic</td>
<td>Organism</td>
<td>Action</td>
<td>Genes/Proteins</td>
<td>Regulation/Expression</td>
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<tr>
<td>Streptomycin</td>
<td><em>Streptomyces griseus</em></td>
<td>Binding to the 16S rRNA of the 30S subunit of ribosome and interfering with the binding of formylmethionyl-tRNA to the 30S subunit.</td>
<td><em>aphD</em> (<em>strA</em>), <em>strK</em>, <em>strV</em></td>
<td>Phosphorylation of streptomycin precursor and efflux pumps</td>
<td>Cotranscription with biosynthetic genes, Regulation by StrR and functional involvement of exporter and phosphotransferase in biosynthesis</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td><em>Streptomyces wedmorensis</em>, <em>Pseudomonas syringae</em> and <em>P. viridiflava</em></td>
<td>Inactivating the enzyme UDP-N-acetylglucosamine-3-enolpyruvyltransferase (MurA) that catalyzes ligation of phosphoenolpyruvate (PEP) to the 3'-hydroxyl group of UDP-N-acetylglucosamine.</td>
<td><em>fomA</em>, <em>fomB</em></td>
<td>Phosphorylation of fosfomycin and efflux pumps</td>
<td>Functional involvement of exporter and phosphotransferase in biosynthesis</td>
</tr>
</tbody>
</table>
| Bleomycin     | *Streptomyces verticillus*        | Inhibiting incorporation of thymidine into DNA strands and inducing DNA cleavage.                         | "blmA and blmB resistant locus" including orf1 to orf7  
(orf2=blmA and orf5=blmB ) | Sequestration and acetylation of bleomycin and efflux pumps | Cotranscription with biosynthetic genes |
| Thioestrepton | *Streptomyces laurantii* and *Streptomyces azureus* | Forming a complex with 23S rRNA and r-protein L11 of 50S subunit and blocking the function of elongation factor EF-Tu and EF-G. | *tnsR*  
(within ribosomal protein operons and outside the thioestrepton gene cluster) | Methylation of 23S rRNA in 50S subunit | Unknown, might be consistently transcribed |
| Ansamycin     | *Amycolatopsis* (rifamycin B, spp.) | Binding β subunit of | *rifP, rhoR*  
(outside rifamycin expression) | Efflux pumps, rifamycin expression | Consistently expression of |
<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
<th>Mechanism</th>
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</thead>
<tbody>
<tr>
<td>Naphthomycin</td>
<td>(A. mediterranei), Streptomyces spp. (S. hygroscopicus) and Nocardia spp.</td>
<td>Prokaryotic RNA polymerase and blocking chain elongation. The rifamycin gene cluster</td>
</tr>
<tr>
<td>Geldanamycin</td>
<td></td>
<td>Resistant RNA polymerase or rifamycin triggering export via RifQ.</td>
</tr>
<tr>
<td>Ansamitocin</td>
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</tr>
<tr>
<td>Streptolydigin</td>
<td>Streptomyces lydicus</td>
<td>Binding ( \beta ) subunit of prokaryotic RNA polymerase and blocking chain elongation. ( slgt, rhoB ) (outside the streptolydigin gene cluster) Efﬂus pumps, streptolydigin resistant RNA polymerase Consistently expression of resistant RNA polymerase or streptolydigin triggering export via SlgR1?</td>
</tr>
<tr>
<td>Kirromycin</td>
<td>(S. cohnus Tü 365, S. cinnamoneus) and Nocardia spp. (N. lactamdurans)</td>
<td>Binding to domain I and II of elongation factor EF-Tu, arresting the EF-Tu+GDP complex in ribosome and blocking elongation cycle. ( tuf ) (outside the kirromycin gene cluster), ( kirTI, kirTII ) Consistently expression of resistant EF-Tu and efﬂus pumps Consistently expression of resistant EF-TU or kirromycin triggering export via KirRI and KirRII?</td>
</tr>
<tr>
<td>Simocyclinone D8</td>
<td>Streptomyces antibioticus Tü 6040</td>
<td>Binding ( \alpha ) subunit of gyrase. Efﬂus pumps Simocyclinone D8/intermediate s triggering export via SimR</td>
</tr>
<tr>
<td>Landomycin A</td>
<td>Streptomyces cyanogenus</td>
<td>Inhibiting DNA synthesis by blocking uptake of thymidine. Efﬂus pumps Cotranscription with biosynthetic genes and landomycin A and its intermediates triggering export via LanK</td>
</tr>
<tr>
<td>Aureolic acid</td>
<td>(chromomycin A3, mithramycin)</td>
<td>Binding G/C rich regions of DNA minor groove and prohibiting DNA replication and transcription. ( cmrA, cmrB, cmrX, or mtrA, mtrB, mtrX ) Efﬂus pumps, UvrA-like protein of ABC nuclease excision system involved in DNA repair Involvement of export in early stage of biosynthesis or chromomycin/intermediates triggering export via CmmRII</td>
</tr>
<tr>
<td>Daunorubicin/Doxorubicin</td>
<td>Streptomyces peucetius</td>
<td>Intercalating DNA and inhibiting DNA replication. ( drrA, drrB, drrC, drrD(?) ) Efﬂus pumps, UvrA-like protein of ABC nuclease excision system Doxorubicin/intermediates triggering resistance and biosynthesis via DnrO-DnrN-</td>
</tr>
</tbody>
</table>
(1) Atypical tetracycline such as anhydrotetracycline and 6-thiatetracycline, etc. disrupts cell membrane.

(2) It was also reported that aminoglycoside could disrupt integrity of cell membrane.
Chapter II

Activation of actinorhodin export by actinorhodin biosynthetic intermediates in *Streptomyces coelicolor*

Adapted from


2.1 Abstract

Actinorhodin is a blue-pigmented antibiotic produced by *Streptomyces coelicolor*. It is believed to be exported by efflux pumps encoded by the *actAB* operon which is negatively controlled by the ActR transcriptional regulator. Normal yield of actinorhodin
depends on the ActA and ActB exporters and this is consistent with previous findings that both actinorhodin and its 3-ring intermediates (e.g. (S)-DNPA) can bind ActR and derepress actAB in vivo. More importantly, an ActR mutant (T142W) that interacts productively with (S)-DNPA but not actinorhodin induces actAB expression resulting in normal yields of actinorhodin. This indicates that actinorhodin biosynthetic intermediates serve as intracellular signals to activate export from producing cells. The mature antibiotic is not the primary signal or not required for this event.

2.2 Introduction

*Streptomyces* are soil dwelling, Gram positive bacteria that are well known for their unusual, complex life cycle. This life cycle commences with spore germination and the growth of vegetative hyphae that extend and branch into a network of substrate mycelia\(^\text{117}\). In response to undefined signals (e.g. possibly nutrient depletion), a second cell type called aerial hyphae starts to break away from substrate mycelium, which concomitantly produce secondary metabolites and initiate programmed cell death\(^\text{118-121}\). In contrast to substrate hyphae, aerial hyphae do not branch but synchronously subdivide into long chains of monogenomic prespore compartments which subsequently round up and metamorphose into mature spores\(^\text{122-124}\). The other important feature of *Streptomyces* is their production of a vast variety of secondary metabolites, including antitumor agents, pesticides and around two-thirds of clinically used antibiotics\(^\text{125, 126}\). Decades of genetic studies on morphological development and secondary metabolism revealed intricate crossregulation between them. Many of the most important insights into the process
involve the model organisms, *S. coelicolor* A3 (2). *S. coelicolor* harbours over 20 gene clusters that are known or predicted to direct production of secondary metabolites\(^{127, 128}\). Among these clusters, the one that encodes the biosynthetic pathway of the blue pigmented polyketide, actinorhodin, has been used as a model to study antibiotic biosynthesis and export for many years\(^{129, 130}\).

### 2.2.1 Actinorhodin biosynthetic gene cluster

Actinorhodin (ACT) has a unique dimeric structure consisting of two benzoisochromanequinone (BIQ) chromophores connected by a C-C bond. It is also a pH indicator, showing red colour under acidic conditions and blue colour under basic conditions\(^{129, 131}\). This property has greatly facilitated the detection of ACT production and the characterization of ACT biosynthetic mutants. The entire ACT biosynthetic pathway resides as a continuous DNA segment spanning over 22 kb on the chromosome\(^{131, 132}\). For historical reasons, this *act* gene cluster was divided into three regions (Figure 2.1): (1) the *actII* region in the middle, carrying the gene encoding the pathway specific activator (*actII-orf4*) and genes for export/resistance (*actII-orf1*, *actII-orf2* and *actII-orf3*)\(^{99, 132-134}\); (2) the right arm of the *act* cluster consists of genes involved in polyketide backbone assembly (genes of polyketide synthase (PKS), *actI-orf1/orf2/orf3*), immediate reduction (ketoreductase gene, *actIII*), aromatization (cyclase/dehydratase gene, *actVII*), cyclization (cyclase gene, *actIV*), and *actVB* at the right border of the cluster which functions in the penultimate biosynthetic step\(^{135-138}\); (3) the left arm of the *act* cluster comprises two groups of genes (*actVI* and *actVA*) which participate in post PKS tailoring steps\(^{138-144}\).
Figure 2.1 Actinorhodin biosynthetic gene cluster

Genes encoding polyketide synthase, tailoring enzymes, regulators and export/resistance system are coded as the indicative patterns. The entire gene cluster consists of three basic regions: center for regulation and efflux/resistance, right arm for backbone formation (actVB is an exception as it involves in late tailoring steps) and left arm for ring modifications.

2.2.2 Assembly of the polyketide skeleton and immediate cyclization

Assembly of the 16-C skeleton of each half molecule of ACT is catalyzed by three separate units of minimal PKS (Figure 2.2): the α and β subunits of β-ketosynthase (KSα and KSβ), encoded by actI-orf1 and actI-orf2 respectively, form a heterodimer with a catalytically inactive β subunit serving as a chain length factor (CLF) that decides the number of condensation cycles. The starter acetate unit is first loaded on to the active cysteine thiol group of KSα, while the first extender malonyl-CoA is connected to a thiol arm of the holo form of acetyl carrier protein (ACP) encoded by actI-orf3. Malonyl decarboxylation is then catalyzed by the acylated KS and the resultant enolate anion on ACP joins the starter acyl unit from the KS. This yields the β-ketoacyl-S-ACP and frees the KS cysteine thiolate for the next 7 elongation cycles (Figure 2.2, step 1 and 2). However, the nascent polyketonic backbone of octaketide undergoes only one reduction on the β-keto group of C9, catalyzed by ketoreductase (KR, encoded by actIII) either during or after the elongation (Figure 2.2, step 3). Other early structural enzymes that are closely associated with the minimal KS include a bifunctional
cyclase/dehydratase encoded by actVII which is involved in aromatization of the first ring by dehydration between C7 and C12, and a cyclase encoded by actIV which catalyzes the second ring formation by aldol condensation between C5 and C14 (Figure 2.2, step 4 and 5)\textsuperscript{131, 135}. The subsequent bicyclic intermediate is subject to the post-PKS modification steps.

\textbf{Figure 2.2} Biosynthesis of actinorhodin polyketide backbone and nascent modification

After 8 cycles of elongation, acetate units are condensed into a 16-carbon linear polyketide (actI-orf1/orf2/orf3) which undergoes immediate reduction, dehydration and cyclization (actIII, actVII and actIV) to generate bi-cyclic intermediate attached to ACP. Genes involved in these early steps are highlighted in the cluster.
2.2.3 Cyclization of the third ring and formation of \((S)\)-DNPA

The first key step is the formation of the third ring, a pyran ring, with correct stereochemical configuration \((3S, 15R)\) (Figure 2.3). Based on earlier studies showing that two \(\text{actVI} \) mutants gave rise to the shunt product aloesaponarin II, genes located within the \(\text{actVI} \) region were deduced to be involved in this step\(^{139, 140, 148}\). The \(\text{actVI} \) region consists of six open reading frames (ORF), the leftward \(\text{orfB} \) and \(\text{orfA} \) and rightward \(\text{orf1} \), \(\text{orf2} \), \(\text{orf3} \) and \(\text{orf4} \). Chemical characterization of gene disruption showed that \(\text{actVI-orf1} \) protein, a \(\beta\)-hydroxyacyl-CoA dehydrogenase (HACD), reduces the keto-group at C3 to provide the chiral secondary alcohol (Figure 2.3, step 1) that would cyclize with the keto-group of C15 to form a hemiketal (Figure 2.3, step 2)\(^{149}\). Enzymatically facilitated by proteins encoded by \(\text{actVI-orfA} \) and \(\text{actVI-orf3} \), a dehydration follows and gives the first tricyclic intermediate, \((S)\)-DNPA (Figure 2.3 step 3)\(^{140, 149, 150}\).
Figure 2.3 Formation of (S)-DNPA

A (S) stereochemical configuration is introduced to the C3 of ACT bi-cyclic intermediate by gene product of actVI-orf1, followed by the cyclization with the keto-group of C15 and a dehydration, forming a pyran ring and the first 3-ring intermediate (S)-DNPA. Genes involved in these steps (actVI-orf1/orf3/orfA) are highlighted in the cluster. Mutations at actVI-orf1 would disrupt the ACT biosynthetic flow at making shunt products DMAC (8) and Aleosaponarin II (3).

2.2.4 Formation of 6-DDHK and DHK

The following stereospecific reduction at C15 proceeds in two coupled steps (Figure 2.4): a reductase encoded by actVI-orf2 carries out reduction of the double bond between C14 and C15, followed by a chemically favoured isomerization assisted by the actVI-orf4
gene product giving rise to the α,β unsaturated carbonyl structure of the second tricyclic intermediate, 6-deoxydihydrokalafungin (6-DDHK) (Figure 2.4, step 1)\textsuperscript{139, 140}.

Late tailoring steps involve ring hydroxylation/oxygenation and dimerization of two tricyclic half molecules, which are catalyzed by the gene products of the \textit{actVA} region\textsuperscript{144}. Though not all of their functions have been characterized, a monooxygenase encoded by \textit{actVA-orf6} resembles several hydroxylases in other type II polyketide gene clusters (e.g. \textit{tcmH} of tetracenomycin gene cluster in \textit{S. glaucescens}) that catalyze oxygenation of a variety of substrates independent of the prosthetic group, metal ion or cofactors associated with molecular oxygen\textsuperscript{143, 151}. It converts the phenolic compound 6-DDHK to the corresponding quinone form by oxidation at C6\textsuperscript{143, 151}. During this process, the 6-DDHK is oriented and stabilized by a hydrogen bond formed between the hydroxyl group at C11 and Trp66 of the monooxygenase ActII-Orf6 (Figure 2.4, step 2)\textsuperscript{143, 151}. Such an intramolecular network of hydrogen bonds enhances deprotonation and the formation of C6 carbanion which reacts with molecular oxygen (Figure 2.4, step 2)\textsuperscript{143, 151}. Via a peroxy intermediate, protonation and a subsequent dehydration give the third tricyclic intermediate, dihydrokalafungin (DHK)\textsuperscript{143, 151}.
A stereo-specific reduction is performed on the double bond between C14 and C15 of \((S)\)-DNPA \((actVI-orf2)\), followed by an isomerization \((actVI/orf4)\) to form 6-DDHK. The monooxygenase encoded by \(actVA-orf6\) converts the 6-DDHK to its quinone form, DHK, by oxidation at C6, which might be supported by the network of hydrogen bonding between the intermediate molecule and the residues of the enzyme. Genes involved in these steps are highlighted in the cluster.

2.2.5 Dimerization of tri-cyclic intermediates and formation of actinorhodin

It is not clear when and how the dimeric ACT ring system forms. As the \(actVB\) mutant produced shunt product kalafungin, a lactone form of DHK (Figure 2.5) (Figure 2.5 step 1), it was suggested that the dimerization of two DHK molecules precede the C8 hydroxylation and be catalyzed by \(actVB\) encoded dimerase\(^{152}\). However, the \(actVB\) gene
product was characterized as a NADH: flavin oxidoreductase which pairs with the actVA-orf5 encoded monooxygenase to catalyze C8 hydroxylation (Figure 2.5, step 2)\textsuperscript{138, 142, 153, 154}.

Dimerization of two reduced molecules of hydroxyl-DHK gives rise to the hexacyclic mature product actinorhodon, but the dimerase activity of ActVB is not confirmed yet (Figure 2.5, step 3)\textsuperscript{155}.

**Figure 2.5 Formation of actinorhodon**

Hydroxylation at C8 and dimerization of two DHK molecules are carried out by a pair of monooxygenase (actVA-orf5) and oxidoreductase (actVB), although other actVA genes may participate in these oxidation/hydroxylation/dimerization steps. An actVB mutant generates the shunt product kalafungin, a lactone form of DHK. actVA-orf5 and actVB are highlighted in the cluster.
2.2.6 Actinorhodin export and resistance in *S. coelicolor*

The major ACT exporter/resistance determinant is encoded by a pair of co-transcribed genes, *actII-orf2* and *orf3*, within the *actII* region, which is negatively regulated by the upstream divergent gene *actII-orf1*. The gene orientation between *actII-orf2/orf3* and *actII-orf1* strikingly resembles the aforementioned *tetA-tetR* resistance system in pathogenic bacteria. The promoter of *actII-orf2/orf3* is strongly repressed by the *orf1* gene product which also represses its own promoter, and both promoters were found to be most active in cultures during ACT biosynthesis. Analogous to the scenario where *tetA* is induced by tetracycline, it is likely that *actII-orf2/orf3* is activated by ACT related molecules. By interacting with and changing the conformation of the Orf1 repressors, these signaling molecules release the repressors from the promoter of *orf2/orf3*, leading to the expression of ACT exporter/resistance. Due to their resemblance of the *tetA-tetR* system, we renamed the two exporter genes, *actII-orf2* and *orf3*, as *actA* and *actB* respectively, and the repressor gene *actII-orf1* as *actR*.

2.2.7 *in vitro* evidence suggested that both actinorhodin and its intermediates can bind ActR and release the repressor from DNA

In previous studies, Dr. Kapil Tahlan constructed an *E. coli* based ActR biosensor and used this to detect putative ActR ligands. He observed a significant and specific induction by supplementing *E. coli* culture with the supernatant of the wild-type *S. coelicolor* culture. This for the first time provided experimental evidence that certain
metabolites produced by *S. coelicolor* were recognized by ActR\(^{66}\). To determine whether these ligands are related to the ACT biosynthetic pathway, an *in vitro* relief of DNA binding by ActR was carried out in the presence of ACT, (S)-DNPA (the first tri-cyclic intermediate), or two shunt products, aleosaponarin II (accumulated in the *actVI-orf1* deletion mutant and maintains the aromatic structure in the 3\(^{\text{rd}}\) ring) and kalafungin (a lactone form of the third tricyclic intermediate DHK and accumulated in an *actVB* deletion mutant)\(^{66}\). ACT, (S)-DNPA and kalafungin could all prevent ActR/DNA interaction at a high concentration (100 µM), whereas aleosaponarin II had no such an activity\(^{66}\). A more intriguing observation was that it was not ACT but (S)-DNPA and kalafungin that abolished ActR/DNA interaction at a low concentration (25 µM), showing that these intermediates were more efficient than ACT at dislodging ActR from the bound DNA\(^{66}\).

We concluded that the export repressor ActR could bind benzoisochromanequinone (BIQ) molecules from ACT biosynthetic pathway, and favoured genuine tricyclic intermediates with pyran ring over the hexacyclic end product ACT or other less related anthroquinone shunt product (e.g. aleosaponarin II)\(^{66}\).

Two basic questions remained to be addressed for the intermediate driven resistance mechanism: First, can ACT biosynthetic intermediates activate export/resistance in living cells? Second, can these molecules accumulate in the cell and serve as the biologically relevant inducers to activate export/resistance? To provide *in vivo* experimental evidence for the first question, I created a series of ACT biosynthetic mutants that block ACT production at various stages of biosynthesis, and tested intermediates accumulated by
these mutants for their ability to activate exporter expression. To distinguish the primary intracellular signals that initiate ACT export/resistance in actinorhodin producing cells, I created an ActR mutant that selectively interacts with only 3-ring intermediates but not the final product. The activation of actAB expression and the wild type yield of actinorhodin in this mutant demonstrated that the 3-ring intermediates are the only requirements for efficient expression of export/resistance. The real-time monitoring of the actAB expression was mostly performed using a luminescent reporter (luxCDABE) which was optimized for use in high G-C content bacteria\textsuperscript{156}. Please refer to Appendix 1 for the construction and testing of this reporter.

2.3 Results

2.3.1 Creation of mutants in the ACT biosynthetic cluster and their effects on biosynthesis

I created four ACT biosynthetic mutants by knocking out genes exclusively involved in different biosynthetic steps and summarized the proposed blockage in Table 2.1: (1) ΔactI-orf1/orf2/orf3, where all three polyketide synthase genes were deleted and ACT biosynthesis was completely eliminated; (2) ΔactVI-orf1, where the HACD that determines the closure and stereospecificity of the pyran ring was lost and conversion of bicyclic intermediate to (S)-DNPA was hampered; (3) ΔactVA-orf5, which is deficient for the last steps of C8-hydroxylation and dimerization and blocked in making DHK or kalafungin. In addition, an actAB (by Catherine Auyeung) and (4) an actR null mutant were also constructed.
<table>
<thead>
<tr>
<th>Mutants</th>
<th>Proposed biosynthetic pathway product</th>
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<tbody>
<tr>
<td>ΔactI-orf1/orf2/orf3</td>
<td>No ACT or intermediates</td>
</tr>
<tr>
<td>ΔactVI-orf1</td>
<td>DMAC/Aloesaponarin (shunt product)</td>
</tr>
<tr>
<td>ΔactVA-orf5</td>
<td>DHK (3-ring intermediate)</td>
</tr>
<tr>
<td>ΔactR</td>
<td>Overproduced ACT</td>
</tr>
<tr>
<td>ΔactAB</td>
<td>No/Reduced ACT</td>
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In the beginning, I set out to confirm the impact of ActA/B and ActR on actinorhodin yield in the wild type *S. coelicolor* strain M145. As shown in Figure 2.6A, the actR mutant produced actinorhodin at a level 4-5 fold higher than that of the wild type. In contrast, extracellular actinorhodin yields were reduced ~5 fold by the actAB deletion. Intracellular actinorhodin yields were also sharply turned down in the actAB mutant compared with that by the wild type (Figure 2.6B), suggesting a complicated role played by the export proteins: they are presumably involved in export but also play an unknown supporting role in actinorhodin biosynthesis. Actinorhodin yields were restored to the wild type level by complementation of each mutation *in trans* (Figure 2.6C). The activation of actAB expression is therefore an important step in actinorhodin production. The residual level of extracellular actinorhodin in the actAB mutant may be due to passive diffusion of actinorhodin out of the cells or the action of another transmembrane pump, possibly ActVA-ORF1 (ActC). Moreover, no ACT production was detected in ΔactVA-orf5 or ΔactVI-orf1 (Figure 2.6A and 2.6B). Instead, a diffusible brownish pigment was observed in cultures grown on solid R2YE medium, demonstrating the accumulation of intermediates or derivative shunt products and that both genes are indispensable for ACT.
biosynthesis (Figure 2.6C)\textsuperscript{142, 149, 157}. In addition, the ectopic expression of actVA-orf5/actVI-orf1 from a constitutive promoter, \textit{ermE}*, or production of actR/actAB under its native promoter, in their cognate mutants complemented ACT production (Figure 2.6C).

**Figure 2.6 Actinorhodin production and genetic complementation of biosynthetic and export mutants**

(A) Extracellular production of actinorhodin. M145 (blue) produces actinorhodin after ~40 hours of culture, strains bearing mutations in \textit{actI} (red), actVA-orf1 (orange) or actVA-orf5 (green) produce no detectable actinorhodin. Strains bearing a deletion of actAB (pink) or actR (black) produced reduced or enhanced levels of actinorhodin respectively. (B) Intracellular production of actinorhodin. Accumulation of actinorhodin in M145 (blue) was observable after 40 hours, while deletion mutants of actI (red), actVA-orf1 (green) and actVA-orf5 (cyan) accumulate no detectable actinorhodin. Strains bearing actAB deletion (purple) produce remarkably reduced actinorhodin. (C) Plate assays showing mutant strains with a control vector or the relevant complementation
plasmid growing on R2YE (for ΔactVI-orf1 and ΔactVA-orf5) or MS (for ΔactR and ΔactAB) media.

2.3.2 Tricyclic molecules produced by actinorhodin biosynthetic mutant can activate actAB

To determine whether 3-ring intermediates from the actinorhodin biosynthetic pathway bind ActR \textit{in vivo} and prevent its interaction with DNA, actAB promoter activity was examined in both the wild type M145 and the aforementioned actinorhodin biosynthetic mutants (refer to Table 2.1): ΔactI, ΔactVI-orf1 and ΔactVA-orf5. A luminescent reporter \textit{luxCDABE} directed by the actAB promoter (P_{actAB}) was introduced into the strains specified above. As shown in Figure 2.7, the P_{actAB}-lux fusion was active in M145 but was inactive in the actI null mutant, in accordance with the requirement of an active actinorhodin biosynthetic pathway for the induction of the export genes. The actVI-1 mutant did not exhibit any reporter transcription either, showing that a pyran ring containing tri-cyclic intermediates and/or mature actinorhodin is required for export initiation. By contrast, the actVA-5 mutant, which is blocked for DHK synthesis\textsuperscript{157}, supported P_{actAB}-dependent luminescence, suggesting that 3-ring intermediates can derepress P_{actAB} \textit{in vivo}. 


Figure 2.7 Promoter activity of actAB in wild type M145 and actinorhodin biosynthetic mutants

Luminescence readings from strains containing a fusion of P_{actAB} to luxCDABE (red) or a control vector (blue), pMU1. Only wild type strain M145 and null mutant ΔactVA-5 that produces 3-ring intermediate exhibit an active promoter of exporter. Strains bearing deletions at genes of polyketide synthase (ΔactI) or gene for forming stereo-specific 3-ring intermediate (ΔactVI-1) are silent in P_{actAB} activities.

2.3.3 ActR mutants that respond productively to (S)-DNPA but not ACT in vivo

To test ligand response in actinorhodin producing cells, I created an ActR mutant which can only productively respond to 3-ring intermediates such as (S)-DNPA but not the mature product actinorhodin so that we can deduce the primary signaling molecules to initiate ActA and ActB dependent actinorhodin export/resistance, either the 3-ring intermediates or the 6-ring actinorhodin. This mutant was created by manipulating the ligand binding ability of ActR (see Appendix 2). Two point mutants, T142W and GTYW (G138Y and T142W) were found to be released from DNA only by binding with (S)-DNPA but not actinorhodin in vitro (Figure A2.6). Also, to measure in vivo response of
ActR to 3-ring and 6-ring molecules, I created a transcriptional reporter in which either the wild type or mutant allele of actR along with the actR-actA intergenic region were fused to luxCDABE such that the lux genes were under the control of actAB promoter (Figure 2.8A) and introduced both reporters into a double mutant in which the actinorhodin polyketide synthase encoding operon actI-orf1/orf2/orf3 and the actR gene had been deleted from the act gene cluster (Figure 2.8A).

Without adding molecules, strains containing the reporters exhibited no luminescence consistent with efficient repression of actAB promoter by a wild type and the ActR mutants in the absence of the actinorhodin biosynthetic pathway (Figure 2.8B). I then added actinorhodin to the reporter strain cultures at concentrations ranging from 10 nM – 1 µM and measured luminescence. Consistent with the behavior of these proteins in vitro, actinorhodin induced luminescence in the wild type actR reporter but not in the two mutant ActR strains (Figure 2.8B). In contrast, when (S)-DNPA was added at the same concentration range, luminescence was induced in both the wild type and mutant reporters (Figure 2.8B), demonstrating a productive interaction of both proteins with this 3-ring intermediate. Time course measurements of this reporter responding to actinorhodin and (S)-DNPA (Figure 2-8C) at 10 nM indicated that derepression by the two ligands took place following ~6 hours in both cases. Again, while the wild type reporter was induced by both ligands the two mutant ActR reporters were only induced by the intermediate (S)-DNPA. This indicates that the T142W and the GTYW mutation alter the ligand responsiveness of ActR such that they interact productively with (S)-DNPA but not actinorhodin. This is consistent with our in vitro results and also confirms that both
(S)-DNPA and actinorhodin can diffuse across the cell wall. As the double mutant GTYW includes the single mutation T142W, for the rest of this project I worked with the single mutant.

Figure 2. ActR responds to externally added actinorhodin and (S)-DNPA
(A) Illustration of a ΔactIΔactR mutant bearing a fusion of P<sub>actAB</sub> to luxCDABE and expressing either wild type ActR or the mutant allele. (B) Reporter strains were incubated with varying concentrations of actinorhodin (top) or (S)-DNPA (bottom) as indicated and the induction of P<sub>actAB</sub> for 8.5 hour incubation with the ligand (right) is compared with that
at 0 hour (left). $P_{actAB}$ controlled by WT ActR can respond to both ACT and ($S$)-DNPA, while the one regulated by mutant ActR only responds to ($S$)-DNPA. (C) Time course response of reporter strains expressing either ActR (purple) or the mutant alleles (T142W (blue) and GTYW (orange)) to 10 nM actinorhodin or ($S$)-DNPA.

2.3.4 Pathway intermediates can activate $actAB$ promoter during actinorhodin biosynthesis

Although the experiments in Figure 2.7 and 2.8 demonstrated that 3-ring pathway intermediates produced in vivo or provided exogenously can relieve repression of $P_{actAB}$ by dislodging ActR in living cells as efficiently as actinorhodin, neither of them addressed the question of whether they accumulate in the cytoplasm during normal actinorhodin biosynthesis and therefore serve as export signals.

To answer this question, I introduced the wild type actR-$P_{actAB}$lux or T142W-$P_{actAB}$lux reporters (as in 2.3.3) into a strain in which the actR gene had been deleted from the actinorhodin biosynthetic gene cluster but the rest of the biosynthetic genes were intact (Figure 2.9A). As expected, the $P_{actAB}$-lux fusion was induced in cells where it was under the control of wild type ActR, reaching a peak at 30-40 hours then dropping after 50 hour to 60 hours (Figure 2.9B). Detectable levels of actinorhodin began to accumulate outside the cells following this induction. Both actinorhodin production/export and the induction of $P_{actAB}$ depended on actinorhodin biosynthesis as both were eliminated by an actI operon deletion (Figure 2.9B). When the same experiment was carried out in a strain where the $actAB$ promoter was under the control of T142W, the result was very similar. $P_{actAB}$ was active within 20 hours of culture growth, peaked thereafter and then began to drop. Actinorhodin accumulation in this T142W strain was the same as the strain bearing
wild type actR. Again the induction of $P_{actAB}$ and production of actinorhodin were both dependent on the actI operon (Figure 2.9B).

There was however, a significant difference in the induction of $P_{actAB}$ when it was under the control of ActR relative to the T142W mutant. In the T142W mutant, total luminescence was reduced by more than an order of magnitude from $\sim9000$ RLU/mg to $\sim120$ RLU/mg. Thus, activation of the T142W-regulated $P_{actAB}$ reporter by biosynthetic intermediates was much less efficient than by the activation of the wild type reporter. Recalling the central role of actAB in actinorhodin biosynthesis (Figure 2.6A), it was therefore striking that even with this greatly reduced $P_{actAB}$ activity, the yield of extracellular actinorhodin was about the same in this mutant strain as in M145. Presumably therefore, even though $P_{actAB}$ is much less active, the expression of the actAB provides sufficient levels of the exporter proteins for a normal yield of extracellular actinorhodin. The experiment in Figure 2.9B was carried out in phosphate limited RG-2 medium, which was used previously however the pattern was essentially the same in four other media\textsuperscript{66,129} (Figure 2.9C).
Figure 2. Actinorhodin intermediates from the pathway induced actAB expression
(in trans expressing ActR or T142W)

(A) Illustration of a ΔactR mutant bearing a fusion of P_{actAB} to luxCDABE and expressing either wild type ActR or the mutant allele T142W in trans to the act gene cluster. (B) Actinorhodin production (dashed lines) and P_{actAB} activity (solid lines) were monitored in reporter strains expressing wild type ActR or the T142W mutant in RG-2 medium. Tests in ΔactR deletion mutant are labeled blue and the control tests in ΔactRΔactI mutant are labeled red. P_{actAB} induction is actinorhodin dependent and similar activation patterns
were detected between WT ActR and T142W mutant controlled $P_{actAB}^{lux}$ fusions, although a significant disparity of promoter intensity was observed. There is no significant difference in the yields of actinorhodin which follows the peak of $P_{actAB}$ activity. (C) Induction of $P_{actAB}$ that is regulated by T142W mutant is reproducible in four other media as indicated.

A concern about the positioning of the reporter, particularly the location of actR gene outside the actinorhodin gene cluster, points out two undesirable effects. First, this requires diffusion of $P_{actAB}$-inducing molecules from the actinorhodin biosynthetic gene locus through the cytoplasm to the heterologous locus where ActR controls $P_{actAB}$. The 3-ring intermediates are significantly less stable than mature actinorhodin, so they might be subject to chemical modification during diffusion thereby reducing the $P_{actAB}^{lux}$ response artificially. Furthermore, I assumed that the ActR proteins encoded by the reporters controlled the expression of the actAB genes in the actinorhodin biosynthetic gene cluster as well as the $P_{actAB}^{lux}$ fusion (Figure 2.10A), but was unsure whether this would be as efficient as regulation of the export genes by the repressor positioned in cis.

To solve these uncertainties, I therefore constructed a second set of strains (Figure 2.10A) in which the T142W allele was inserted, in place of wild type actR, into the actinorhodin biosynthetic gene cluster in an otherwise normal M145 strain. I then introduced a fusion of $P_{actAB}$ to luxCDABE (without upstream actR) into mutant and wild type strains and compared the activation of luminescence and production of actinorhodin. The $P_{actAB}$ signals were lower both under the control of wild type ActR and under the control of the T142W protein. However, aside from this, the result was essentially the same as those reported above (compare Figure 2-9B and 2-10B). The yields of
actinorhodin were the same in both strains (and the same as in the first set of reporter strains) regardless of whether export was regulated by wild type ActR or T142W and the timing of induction of the $P_{actAB}$-$lux$ fusion was the same as well except that it was significantly reduced in the T142W strain. Since the T142W allele does not interact productively with actinorhodin, these results demonstrate that sufficient 3-ring intermediates accumulate during biosynthesis to induce the expression of the $actAB$ genes.

Figure 2.10 Actinorhodin intermediates from the pathway induced $actAB$ expression (in cis expressing ActR or T142W)

(A) Illustration of the in situ $actR$ mutant T142W bearing a fusion of $P_{actAB}$ to luxCDABE. (B) Actinorhodin production (dashed lines) and $P_{actAB}$ activity (solid lines) were monitored in strains in cis expressing wild type ActR or the T142W mutant. Similar to that indicated by Figure 2.9, induction of $P_{actAB}$ was detected in both types of strains except for a reduced activity in T142W mutant. The yields of extracellular actinorhodin were also similar in two strains.
2.3.5 The exporter gene was expressed in ActR mutant T142W

Finally, to confirm that our results applied to the actAB genes as well as the \( P_{actAB-lux} \) fusion I carried out RT-PCR using primers directed against the actA coding sequence (Figure 2.11). Again, the results were in agreement with the lux reporter expression, showing that the actA transcript was produced in the strain expressing wild type actR and in the T142W mutant and that induction was dependent on the actI operon and therefore actinorhodin biosynthesis.

![Figure 2.11 RT-PCR analysis of actA expression](image)

**Figure 2.11 RT-PCR analysis of actA expression**

RT-PCR analysis of RNA isolated from M145, an in situ T142W mutant and an actI null mutant 46 and 52 hours after inoculation. Panel A shows the products from a control experiment in which the reverse transcription step was omitted. Panel B shows a positive control experiment using primers directed against the hrdB housekeeping sigma factor encoding gene. Panel C shows the results of RT-PCR using primers directed against the actA gene. Reverse transcript depends on the presence of the actinorhodin polyketide synthase but occurs in both M145 and the T142W mutant.

2.4 Discussion

The fact that there is no difference in actinorhodin yields regardless of the difference in actAB expression when controlled by wild type or T142W mutant ActR suggests that the 3-ring intermediates from the biosynthetic pathway are sufficient for actAB activation.
and this intermediate-driven signalling is the primary or exclusive determinant of initial actAB expression. This prior activation of the efflux pump operon would protect cells from self-killing. Furthermore, the expression of pumps would conversely have influence over actinorhodin biosynthesis. There are two possible mechanisms by which the pumps facilitate biosynthesis.

First, the pump could simply remove the end product to prevent the product inhibition of the biosynthetic enzymes. End product inhibition is common in primary biochemical pathways, such as amino acid biosynthesis or glycolysis, where the final product (e.g. amino acid or ATP) reacts with the first or prior enzymes that are unique to the pathway and causes a conformational change so that the enzyme can no longer interact with its substrate (the intermediates of the pathway). Lacking substrates for the subsequent steps of the pathway, the cell therefore shuts down the biosynthesis of the molecule. Although we have no idea how this negative feedback could occur in antibiotic biosynthesis, our results demonstrate an intimate relationship between actinorhodin production and export: molecules from the biosynthetic pathway are essential for actAB induction and any changes in actAB expression would critically affect yield of actinorhodin (Figure 2.6A).

Second, efflux pumps that are expressed in response to intermediate molecules might serve as a scaffold for assembly of a multiprotein complex of the actinorhodin polyketide synthase and tailoring enzymes. Such a complex could be required for high level production of the end product molecule and would have the added advantage of facilitating direct actinorhodin export at the end of the pathway to promote efficient
export of the end product. Such a coordination of polyketide biosynthesis and transport
via interactions between synthase and cognate pumps was reported in *Mycobacterium
tuberculosis*: PpsE, one of the synthase enzymes for the virulence polyketide lipid
phthiocerol dimycocerosate (PDIM), interacts with a domain of the PDIM transporter,
MmpL7\textsuperscript{159}. It is unknown whether or how ActA and ActB could facilitate the formation
of a multi-enzyme complex of biosynthesis and export in *S. coelicolor*, but predictions of
ActA and ActB topology provide us some intriguing clues for further investigation. The
ActA transmembrane protein belongs to the Major Facilitator Superfamily (MFS) of
efflux pumps which are single-polypeptide secondary carrier that transport small solute in
response to chemiosmotic ion gradients\textsuperscript{62}. It possesses a succession of 12 transmembrane
regions and an extra C-terminal hydrophobic domain separated from the set of prior 12
domains by a long hydrophilic region (Figure 2.12A)\textsuperscript{132, 133}, which is unique in
arrangement (13 transmembrane domains) as MFS typically have 12 or 14
transmembrane domains. In addition, ActB shows conserved domains that are analogous
to MmpL proteins and belong to the Resistance-Nodulation-Cell Division (RND)
Superfamily. The RND efflux system typically consists of an inner membrane protein (e.g.
an ABC transporter ATPase), an outer membrane porin/channel protein and an adapter
protein that bridges the two membrane components\textsuperscript{132, 133}. Therefore, it is usually found in
Gram-negative bacteria. In Gram-positive *S. coelicolor* which doesn’t have an outer
membrane, the translational coupling of *actB* with *actA* suggests a functional connection
between the two. ActB possesses 12 transmembrane helices. Two long hydrophilic
extracellular regions separates the 2\textsuperscript{nd} transmembrane domain from the 1\textsuperscript{st} one, and 8\textsuperscript{th}
from 7th, respectively (Figure 2.12B)\textsuperscript{132, 133}. Although it does not show any pattern for the membrane associated protein nor any typical ATP-binding site, it was suggested that the N terminus of ActB interacts with the C terminus of ActA via the hydrophilic connectors to form an active transport system in which ActB might bind and pass actinorhodin through the export channel provided by the first 12 transmembrane domains of ActA\textsuperscript{132, 133}. It was also suggested that in the process of export, ActB catalyzes the biosynthesis of γ-actinorhodin, the major extracellular form of actinorhodin\textsuperscript{66, 132, 133}.

Deletion of \textit{actA} or \textit{actB} was neither lethal, nor abolished ACT biosynthesis but delayed the appearance of the blue pigment outside the cell. In young cultures of these mutants, the acidic form of ACT (red pigments) was found strictly confined to the mycelium, implying that co-expression of \textit{actAB} is essential for an efficient actinorhodin export and/or biosynthesis\textsuperscript{129}. There was still low level production of actinorhodin when we knocked out both \textit{actA} and \textit{actB} genes (Figure 2.6A). This reduced ACT may be facilitated by a third transporter encoded by \textit{actVA-orf1} (\textit{actC}). ActC is also a MFS transporter with 14 transmembrane helices (Figure 2.12C). A triple knock out mutant of all three transporter genes is under construction by one of our graduate students. If these proteins are only in charge of transport, the triple deletion would be lethal to the cell. But more likely, ActA, ActB and ActC pumps are biochemically involved in actinorhodin biosynthesis and ablation of all three may completely shut down the biosynthetic pathway.
Figure 2. Topology predictions of actinorhodin export proteins

(A) ActA. (B) ActB. (C) ActC. The topological structure of each transporter protein is predicted by a transmembrane helix prediction server TMHMM 2.0. The transmembrane domain is in red, cytoplasmic regions in blue and extracellular regions in pink. The y axis indicates the probability of location and orientation of transmembrane helices in the sequence, while the x axis indicates the positions of helices in the sequence length.
2.5 Conclusion

By testing actAB expression in actinorhodin biosynthetic mutants, I showed that tricyclic intermediates could turn on ACT export in vivo. Furthermore, to demonstrate that these intermediates are accumulated in actinorhodin producing cells and essential for the initiation of export, I created an ActR mutant that responded productively only with (S)-DNPA but not actinorhodin. actAB expression was detected in the “intermediate only” ActR mutant and provided a wild type yield of actinorhodin. These experiments demonstrated that the 3-ring intermediates from actinorhodin biosynthetic pathway are the primary and exclusive intracellular signals to activate ActA and ActB exporter. To understand how the expression of actinorhodin efflux pumps is coordinated across the whole S. coelicolor community and the function of the secreted mature product, we sought to identify the intercellular signaling.

2.6 Materials and methods

Table 2. Bacterial strains and plasmids used in the chapter

<table>
<thead>
<tr>
<th>Strain/plasmid/cosmid</th>
<th>Description/function</th>
<th>Source/Creator</th>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td><em>S. coelicolor M145</em></td>
<td>Wild type host strain, SCP1’, SCP2’</td>
<td>John Innes Centre</td>
</tr>
<tr>
<td><em>S. coelicolor ΔactI</em></td>
<td>M145, ΔactI deletion mutant</td>
<td>Ye Xu</td>
</tr>
<tr>
<td><em>S. coelicolor ΔactVI-1</em></td>
<td>M145,ΔactVI-1 deletion mutant</td>
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<td><em>S. coelicolor ΔactVA-6</em></td>
<td>M145,ΔactVA-6 deletion mutant</td>
<td>Ye Xu</td>
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<td><em>S. coelicolor ΔactVA-5</em></td>
<td>M145,ΔactVA-5 deletion mutant</td>
<td>Ye Xu</td>
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<td><strong>Δspc</strong></td>
<td>M145, actI-orf1/2/3 replaced by spcR cassette</td>
<td>Ye Xu</td>
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<td><em>S. coelicolor ΔactIΔactR</em></td>
<td>M145, ΔactIΔactR deletion mutant</td>
<td>Ye Xu</td>
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<td><em>S. coelicolor ΔactVA-5</em></td>
<td>M145, ΔactVA-5 deletion mutant</td>
<td>Ye Xu</td>
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<tr>
<td><em>E. coli T142W</em></td>
<td>Host strain for DNA conjugation, containing the non-transmissible oriT mobilizing plasmid</td>
<td>Kieser et al. (2000)</td>
</tr>
<tr>
<td>ET12567/pUZ8002</td>
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<tr>
<td>Strain</td>
<td>Characteristics</td>
<td>Source</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------------------------------------------------------</td>
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<td><strong>E. coli BW25113</strong></td>
<td>Host strain for Redirect mutagenesis</td>
<td>John Innes Centre</td>
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<tr>
<td><strong>E. coli DH5α</strong></td>
<td>Host strain for DNA cloning</td>
<td>Strategene</td>
</tr>
<tr>
<td><strong>E. coli XL1-Blue</strong></td>
<td>Host strain for DNA cloning</td>
<td>Strategene</td>
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### Plasmids

<table>
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<tr>
<th>Type</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLASMID pMU1</td>
<td>Promoterless luxCDABE operon, flanked by transcriptional terminators, preceded by in-frame stop codons and ribosome binding sites, aac(3)IV</td>
<td>Craney et al. (2007)</td>
</tr>
<tr>
<td>pMU1-actABlux</td>
<td>Promoterless lux reporter directed by actAB</td>
<td>Ye Xu (2007)</td>
</tr>
<tr>
<td>pWT+IGR+lux</td>
<td>pMU1, actR fused with act operator directed lux reporter (apr^R)</td>
<td>Ye Xu (2007)</td>
</tr>
<tr>
<td>pT142W+IGR+lux</td>
<td>pMU1, actR&lt;sup&gt;T142W&lt;/sup&gt; fused with act operator directed lux reporter (apr^R)</td>
<td>Ye Xu (2007)</td>
</tr>
<tr>
<td>pGTYW+IGR+lux</td>
<td>pMU1, actR&lt;sup&gt;G138Y,T142W&lt;/sup&gt; fused with act operator directed lux reporter (apr^R)</td>
<td>Ye Xu (2007)</td>
</tr>
<tr>
<td>pOJ260</td>
<td>Conjugatable Streptomyces suicide plasmid (apr^R)</td>
<td>Bierman et al. (1992)</td>
</tr>
<tr>
<td>pOJ260-T142W</td>
<td>For preparing the <em>S.coelicolor</em> actR&lt;sup&gt;T142W&lt;/sup&gt; point mutant (apr^R)</td>
<td>Ye Xu (2007)</td>
</tr>
<tr>
<td>pOJ260-GTYW</td>
<td>For preparing the <em>S.coelicolor</em> actR&lt;sup&gt;G138Y,T142W&lt;/sup&gt; double point mutant (apr^R)</td>
<td>Ye Xu (2007)</td>
</tr>
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<td>PIJ773</td>
<td>Template plasmid for apramycin resistance disruption cassette in Redirect mutagenesis aac(3)IV, amp</td>
<td>John Innes Centre</td>
</tr>
<tr>
<td>PIJ778</td>
<td>Template plasmid for spc&lt;sup&gt;R&lt;/sup&gt; disruption cassette in Redirect mutagenesis (spc&lt;sup&gt;R&lt;/sup&gt;, amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>John Innes Centre</td>
</tr>
</tbody>
</table>
Expression of protein for DNA conjugation in trans, kan

Kieser et al. (2000)

Bacterial artificial chromosome containing act

John Innes Centre

gene cluster

amp\(^b\), ampicillin resistant; apr\(^a\), apramycin resistant; cam\(^c\), chloramphenicol resistant; kan\(^d\), kanamycin resistant; spc\(^e\), spectinomycin resistant

Table 2.3 Oligonucleotide primers used in this chapter

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′→3′)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^a)actABpF</td>
<td>TCGTCG GCTTTCAACGGAACTCATGG</td>
<td>Forward primer for actAB promoter cloning</td>
</tr>
<tr>
<td>actABpR</td>
<td>CTTGCACGGACCTCTCTACGTA</td>
<td>Reverse primer for actAB promoter cloning</td>
</tr>
<tr>
<td>KOactI-F</td>
<td>AGCCCGGTAGGGACCCACGAGGCCAGGG</td>
<td>Forward primer for actI deletion by Redirect</td>
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<td></td>
<td>GAGGGGAACACATGATTCGGGGGAT</td>
<td>mutagenesis</td>
</tr>
<tr>
<td></td>
<td>CCGTCGACC</td>
<td></td>
</tr>
<tr>
<td>KOactI-R</td>
<td>GGGTGTGAGGACCACCCGTTGTTCCTC</td>
<td>Reverse primer for actI deletion by Redirect</td>
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<td>GGCAGGCTCATGG</td>
<td>mutagenesis</td>
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<td></td>
<td>TGTAGGCTGGAGCTGCTTC</td>
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<tr>
<td>KOactVI-1F</td>
<td>AGCCCGGTTCCGAGTGGGCGCTGCCA</td>
<td>Forward primer for actVI-orf1 deletion by</td>
</tr>
<tr>
<td></td>
<td>GGCTCGTCGCCATG</td>
<td>Redirect mutagenesis</td>
</tr>
<tr>
<td></td>
<td>ATTCGGGGATCCCGTCGAC</td>
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<tr>
<td>KOactVI-1R</td>
<td>GGGTCCCCGTACCCGATCCGTCACTGCGAGC</td>
<td>Reverse primer for actVI-orf1 deletion by</td>
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<td></td>
<td>GCACGATCATGCTT</td>
<td>Redirect mutagenesis</td>
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<td></td>
<td>TGTAGGCTGGAGCTGCTTC</td>
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<td>KOactVA-6F</td>
<td>ACGCCGGAGAGCTGTTGAGGAGGAG</td>
<td>Forward primer for actVA-orf6 deletion by</td>
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<tr>
<td></td>
<td>CAAGATGGCTGCAA</td>
<td>Redirect mutagenesis</td>
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<td></td>
<td>ATTCGGGGATCCCGTCGAC</td>
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<tr>
<td>KOactVA-6R</td>
<td>AGGTCAAGCGGACCGAGGATCGCCCC</td>
<td>Reverse primer for actVA-orf6 deletion by</td>
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<td></td>
<td>CCGGAGATCATGAA</td>
<td>Redirect mutagenesis</td>
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<tr>
<td></td>
<td>TGTTAGGGCTGGAGCTGCTTC</td>
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<tr>
<td>KOactVA-5F</td>
<td>GGCACGGACCGGTGAGGACCGGG</td>
<td>Forward primer for actVA-orf5 deletion by</td>
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<td></td>
<td>GACCGCCGATGAC</td>
<td>Redirect mutagenesis</td>
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<tr>
<td></td>
<td>ATCCGGGGATCCCGTCGAC</td>
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<tr>
<td>KOactVA-5R</td>
<td>GCGACGAGGCCACCCCGGGGTGAG</td>
<td>Reverse primer for actVA-orf5 deletion by</td>
</tr>
<tr>
<td></td>
<td>TCACTTCAGACCATC</td>
<td>Redirect mutagenesis</td>
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<td></td>
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<tr>
<td>KOactRR</td>
<td>TGTTCATGACTCCGGGCGGATCG</td>
<td>Reverse primer for actR deletion by Redirect</td>
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<tr>
<td></td>
<td>CTGCACCTGCAGAC</td>
<td>mutagenesis</td>
</tr>
<tr>
<td></td>
<td>TGTAGGCTGGAGCTGCTTC</td>
<td></td>
</tr>
<tr>
<td>(^a)actABpF</td>
<td>TCGTCGTCCTAGA</td>
<td>Forward primer for generating (P_{actR-lux})</td>
</tr>
<tr>
<td></td>
<td>GCTTTCAACGGAACTCATGG</td>
<td></td>
</tr>
</tbody>
</table>
2.6.1 Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids/cosmids involved in this chapter were listed in Table 2.2. Plasmids/cosmids were propagated in *Escherichia coli* strain XL1-blue/DH5α,
which were grown at 37 °C in Luria Broth (LB) medium. Antibiotic concentrations for LB were: 100 µg/ml of ampicillin, 50 µg/ml of kanamycin, 50 µg/ml of apramycin, 25 µg/ml of chloramphenicol, 50 µg/ml spectinomycin and 100 µg/ml carbenicillin. For introduction of plasmids/cosmids into *Streptomyces coelicolor*, plasmids/cosmids were first transformed into non-methylating *E. coli* strain ET12567/pUZ8002 allowing for conjugal transfer of DNA. *S. coelicolor* strains were grown on solid media R2YE or MS agar at 30 degree, or grown in liquid media ACT production medium (APM) at 30 °C shaking at 200 rpm. Antibiotic concentrations for *Streptomyces* media were 50 µg/ml apramycin, 50 µg/ml kanamycin, 400 µg/ml spectinomycin and 25 µg/ml nalidixic acid.

### 2.6.2 Procedures for DNA manipulation

Oligonucleotides used in this chapter are listed in Table 2.3. Standard procedures were used for plasmid/cosmid DNA isolation, subcloning and analysis (Sambrook et al. 1989; QIAGEN). Polymerase chain reaction (PCR) was performed using Vent® DNA polymerase (NEB) and all PCR products were sequenced. Manipulation on cosmid DNA employed established protocols (Kieser et al. 2000).

### 2.6.3 Construction of actinorhodin biosynthetic and export mutants

The actinorhodin biosynthetic and export mutants Δ*actI*, Δ*actVI*-1, Δ*actVA*-6, Δ*actVA*-5, Δ*actAB* and Δ*actR* were prepared by PCR targeting system in *S. coelicolor* (ReDirect Manual, John Innes Centre).

The first step is to replace the target gene within a *S. coelicolor* cosmid (Redenbach et al. 1996) by a disruption cassette which consists of a selectable marker (e.g. apr^R^).
encoded by *aac(3)IV* and other elements (e.g. *oriT* and Flipase Recognition Target (FRT)): First, the disruption cassette is amplified from corresponding template plasmid (e.g. pIJ773) by PCR using primers with 39 nt extensions homologous to the up/downstream sequences of the target gene. Second, both the disruption cassette with overhangs of homology and the cosmid bearing the target gene (e.g. BAC28G1 containing the *act* gene cluster) are introduced *E. coli* strain BW255113 containing pIJ790 allowing homologous recombination occurring between the linear cassette and the cosmid, resulting in a “PCR targeted” cosmid that has the target gene replaced by the cassette.

Then, the targeted cosmid is introduced into *S. coelicolor* by conjugation (Matsushima et al. 1994), and the disruption cassette on the cosmid replaces the target gene on the chromosome via successive homologous recombinations, giving a *Streptomyces* disruption mutant with a chromosomal selectable marker. Meanwhile, the targeted cosmid is transformed into *E. coli* strain DH5α holding plasmid BT340. The Flipase system expressed by BT340 recognizes FRT sites and excises the flank disruption cassette, leaving an 81 nt scar. This “clear” cosmid is introduced into the previously created disruption mutant by protoplast transformation and removes disruption cassette by homologous recombinations, giving a clear knock out mutant with in frame deletion on target gene. Disruption cassette with spectinomycin<sup>R</sup> marker was amplified from pIJ778 and used for creating ΔactI and ΔactR mutant; while cassette of apramycin<sup>R</sup> marker was amplified from pIJ773 and used for other mutants. Primers to amplify disruption cassette are listed in Table 2-3: ΔactI: KOactIF and KOactIR; ΔactVI-1: KOactVI-1F and
KO\textit{actVI}-1R; \textit{\Delta}\textit{actVA}-6: KO\textit{actVA}-6F and KO\textit{actVA}-6R; \textit{\Delta}\textit{actVA}-5: KO\textit{actVA}-5F and KO\textit{actVA}-5R; \textit{\Delta}\textit{actR}: KO\textit{actRF} and KO\textit{actRR}. FLP Recognition Target (FGT) sequences are underlined.

An \textit{\Delta}\textit{actR}\textit{\Delta}\textit{actI} double mutant was built on an \textit{S. coelicolor} \textit{actI}::\textit{spcR} strain by knocking \textit{actR} using the same PCR targeting system (ReDirect mutagenesis, Gust et al.).

2.6.4 Luminescent assays for actinorhodin biosynthetic and export mutants

Oligonucleotide primers listed in Table 2.3 were used to amplify \textit{act} promoters that consist of the whole inter-genetic region by PCR from BAC28G1 cosmid. The fragment was ligated into BamHI/XbaI and KpnI sites of pMU1, giving rise to a luminescent reporter directed by \textit{act} promoter (e.g. pMU1-\textit{actABp}\textit{lux}). This integrating plasmid was introduced into wild type \textit{S. coelicolor} strain M145 or various ACT biosynthetic mutants by conjugation. Exconjugants were selected by apramycin (50 µg/ml) and nalidixic acid (25 µg/ml), and restreaked on R2YE agar/apramycin (50 µg/ml) for making spore stocks.

For luminescent tests, \textit{S. coelicolor} spores at initial concentration \(\approx 0.5\times10^6\) spores/ml were inoculated in APM liquid medium. Two hundred microlitres of each was taken every day for at least three days and measured using Lumat 9507 luminometer (Bertholt Technologies).

2.6.5 Construction of luminescent reporter regulated by WT \textit{ActR} or T142W mutant allele

We constructed two sets of \textit{luxCDABE} reporters for monitoring \(\textit{P}_{\textit{actAB}}\) activity. First we generated an \textit{actAB} promoter fragment using primers \textit{actABp}\textit{F} and \textit{actABpR} (Table
2-3) and inserted it between the XbaI and KpnI sites of pMU1 (notice that forward primers include XbaI restriction site instead of BamHI site as demonstrated for building P_{actAB-lux} reporter), giving rise to pMU1-actABplux. Second, we generated reporters where actR/actR* is fused with the actAB promoter directed lux genes, WT actR, actR^{T142W} and actR^{GTYW} were amplified from pTOPO-actR, pET151-T142W and pET151-GTYW respectively by primers AR/F and AR/R (see Table 3-4). The generated fragments were ligated into BamHI and XbaI cut pMU1-actABplux to give pWT+IGR+lux, pT142W+IGR+lux and pGTYW+IGR+lux.

pMU1-actABplux was introduced into M145 and two actR in situ replaced mutants, actR^{T142W} and actR^{GTYW}, by conjugation. The other set of luxCDABE reporter, pWT+IGR+lux, pT142W+IGR+lux and pGTYW+IGR+lux, was introduced into ΔactRΔactI or ΔactR knock out mutant, by conjugation. Exconjugants were selected by apramycin (50 μg/ml) and restreaked on R2YE agar for preparing spore stocks.

S. coelicolor ΔactIΔactR strains consisting of luminescent reporter for external ligand feeding assays were inoculated into R2 liquid medium and grown overnight until OD_{450} ≈ 0.1. ACT or (S)-DNPA pure compounds (dissolved in DMSO) were added to overnight culture to a series of final concentrations: ACT, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, 250 nM, 500 nM, 750 nM, 1 μM; (S)-DNPA, 10 nM, 25 nM 50 nM, 75 nM, 100 nM, 250 nM, 500 nM, 750 nM, 1 μM. DMSO was added as a negative control. All cultures were kept incubating and shaking at 30°C, 200 rpm for another 8.5 hours, and 200ul of each was taken for luminescent readings at time point 0, 3, 6, 7, 7.5 and 8.5 hour, and measured using Lumat 9507 luminometer (Bertholt Technologies).
Other *Streptomyces* consisting of luminescent reporter or the promoterless pMU1 were inoculated in R2YE liquid medium at $0.5 \times 10^6$ spores/ml and grown at 30°C with shaking at 200 rpm until OD$_{450}$ reached approximately 0.1. Cells were recovered by centrifugation at 3000 rpm, washed once with 0.85% saline and resuspended in RG-2 (or other) medium and incubated at 30 °C with shaking at 200 rpm. The luminescence assays were measured using Lumat 9507 luminometer (Bertholt Technologies). The luminescent readings and actinorhodin assays were taken as a function of time and normalized to the wet weight of cell pellet.

### 2.6.6 Determination of ACT production in *S. coelicolor*

Total ACT content was determined by treating 200μl of each culture with KOH (1N final concentration), centrifuging, and measuring supernatant at A640 by plate reader (Epoch, Biotech) (Practical *Streptomyces* Genetics, John Innes Centre).

### 2.6.7 Total RNA extraction

M145, T142W and ΔactI were inoculated into R2YE liquid culture at $5 \times 10^6$ spores/ml and grown overnight until mid-exponential phase (OD$_{600}$$\approx$0.4-0.6). Cells were harvested and subcultured into RG-2 liquid medium as we did for luminescent assays, and continued growing for three days. 15 ml cells were harvested at 46 hour and 52 hour after inoculation and homogenized with in 5 ml modified Kirby Mix (1% (w/v) N-lauroyl-sarcosine, 6% (w/v) sodium 4-aminosalicylate and 6% (v/v) phenol in 50 ml Tris-Cl, pH 8.3) and lyzed by vortexing 2min in presence of 4 beads. Total nucleic acids were removed from Cell debris and proteins by extraction with 5 ml acidic phenol: chloroform:
Isoamyl alcohol (25:24:1) (Invitrogen) twice and precipitated from the aqueous phase by adding 1/10 volume 3 M sodium acetate (pH 6.0) and 1 volume Isopropanol. The pellet was washed by 95% ethanol and redissolved in 450μl RNase free water. The nucleic acids were treated with RNase free DNase I and incubated at 37°C to remove chromosomal DNA. The RNA was extracted with equal volume of acidic phenol: chloroform: isoamyl alcohol (25:24:1) (Invitrogen) and precipitated by 3 M sodium acetate and isopropanol as mentioned above. The pellet was washed by 80% ethanol, air dried and dissolved in 50-100 μl RNase free water. The quantity and quality of purified RNA were analyzed by spectrophotometer. The A260/A280 for high qualitative RNA is between 1.9- 2.05. The integrity of RNA was analysed by running ~1μg sample on 1% agarose gel. Both 23S and 16S rRNA bands were visible and the absence of top band indicated no DNA contamination.

2.6.8 Reverse Transcription PCR (RT-PCR)

Purified RNA samples (1μg) were applied as an initial template in RT-PCR using QIAGEN One-step RT-PCR kit containing enzyme blend and unique formulated buffer for both reverse transcription and PCR and following manufacturer’s protocol. *actAB* gene specific primers (*actAB/F* and *actAB/R*, Table 2.3) would generate a 301bp product amplified from cDNA. To check DNA contamination, regular PCR was running by skipping initial reverse transcription reaction. And *hrdB* gene specific primers that would generate a 350 bp PCR product were used to check consistency of RT-PCR.
Chapter III

Actinorhodin acts as an intercellular signaling molecule to activate actinorhodin resistance across the *S. coelicolor* community

Adapted from

3.1 Abstract

Actinorhodin-producing cells can induce the actAB promoter in non-producing cells and this intercellular signaling event requires mature actinorhodin. Furthermore, while the ‘intermediates responsive only’ ActR mutant permits sufficient actAB expression for normal actinorhodin yields, its inability to respond to extracellular actinorhodin leads to widespread cell death. This demonstrates that a second intercellular signaling event mediated by actinorhodin is required for the establishment of resistance throughout the culture.

3.2 Introduction

In previous studies, I found that the actAB encoded exporter was an important determinant of actinorhodin yield. Expression of this efflux pump is closely coordinated with actinorhodin biosynthesis and mediated by a TetR family regulator, ActR. Previous in vitro experiments showed that ActR could interact with both actinorhodin and its pathway intermediates and be dislodged from actAB promoter so as to turn on expression of the transporter, and we suggested that 3-ring intermediates, such as (S)-DNPA might serve as the real signaling molecules to initiate export/resistance\(^6\). Induction of actAB promoter in actinorhodin biosynthetic mutant blocked at making 3-ring intermediate (DHK) suggested that these molecules could function as in vivo inducers. To distinguish induction led by intermediates from that by final product, I created an ActR mutant that could productively interact with only 3-ring intermediates ((S)-DNPA) but not actinorhodin (See Appendix 2) and found it responded to the actinorhodin biosynthetic
pathway in vivo with actAB induction and normal yield of actinorhodin. All these observations are consistent with the view that actinorhodin intermediates act as preliminary signals to turn on export/resistance genes and this alone produces sufficient expression of efflux pumps to translocate mature products out of the cell.

However, we were concerned about the discrepancy between the activation of P\textsubscript{actAB} regulated by wild type and the mutated ActR\textsuperscript{T142W} (Figure 2.9 and 2.10) and wondered if there might be another component in the actAB activation process that depends on actinorhodin itself. For example, perhaps within S. coelicolor communities, some cells produce actinorhodin before all the others or, alternatively, perhaps there are specialized actinorhodin producing and non-producing cell populations. If so, then it might be advantageous if the colony could induce the export genes in all cells rather than exclusively in the actinorhodin producing cells. While intermediates are sufficient for the derepression of actAB in actinorhodin producing cells, there might be a second intercellular signaling mechanism mediated by actinorhodin itself which is for the self-protection from neighboring actinorhodin producers. Is it possible that the secreted actinorhodin can activate expression of actAB exporter in sibling cells and coordinate the export/resistance across the culture? The T142W mutant that responds only to actinorhodin tri-cyclic intermediates provided us an experimental tool to study how the intercellular communication was carried out among Streptomyces colonies.

As shown by a simplified model, in wild type culture (Figure 3.1A), actinorhodin biosynthesis is started in one cell (or one colony), which turns on actAB expression and leads to the export of actinorhodin (and possibly a limited amount of intermediates).
These 6-ring molecules diffuse into neighboring cells where they are recognized by ActR and derepress the expression of ActA and ActB exporter which would be reinforced once the endogenous actinorhodin biosynthesis is started. In a T142W mutant culture (Figure 3.1B), intermediates activate ActA and ActB in the cell (or colony) which turn on actinorhodin production earlier than the cohorts and the secreted actinorhodin has the same opportunity to enter neighboring siblings. However, the ActR mutant only responds to the 3-ring intermediates which are confined inside the producing cell or cannot work effectively as extracellular signaling molecules, resulting in the rest of the culture remaining uncommunicative.

Figure 3.1 Hypothetical models of intercellular signaling in wild type or T142W mutant cultures via actinorhodin related molecules
Each square represents a cell or a colony. Cells/colonies that express ActA and ActB transporter are coded blue. In the absence of signals, actAB expression (demonstrated by P_\text{actAB-lux}) is repressed by either wild type ActR (A) or T142W (B). Early actinorhodin producing cell (top left blue square) relieves this repression first and produces signals which make access to the neighboring cells. (A) Wild type cells will respond productively to these external signals and release ActR from P_\text{actAB}; while (B) T142W mutant cells remain inactive in turning on actAB expression.
To test this hypothesis, I ran a mixed culture experiment by growing actinorhodin non-producing mutant (ΔactI/ P\(_{actAB}\)-lux) with donor strains that produce actinorhodin or its 3-ring intermediates and detected how P\(_{actAB}\) responded to these two types of intercellular signals when it was under control of either wild type or T142W mutated ActR. I also attempted to capture the induction of actAB expression across the wild type or T142W mutant cultures by monitoring the expression of P\(_{actAB}\) directed eGFP. Both approaches indicate that the mature product, actinorhodin (or its extracellular form γ-actinorhodin) is the exclusive intercellular signal that triggers on the export/resistance across the bacterial community of \(S.\ coelicolor\). Mutant (e.g. T142W) that fails in establishing this intercellular signaling will eventually pay the price: death.

3.3 Results

3.3.1 Actinorhodin acts as an exclusive intercellular signal

In order to test this hypothesis, it was necessary to determine whether actinorhodin or its biosynthetic intermediates can act as intercellular signals that activate P\(_{actAB}\). I carried out mixed culture experiments where one strain served as the donor of inducing molecules and an ΔactRΔactI double null mutant harboring an P\(_{actAB}\)-lux reporters as the recipient to measure actAB expression under the control of either wild type ActR or ActR\(^{T142W}\) (Table 3.1). If both types of the signaling molecules can be secreted out of the producing cells, recipient strain expressing the wild type ActR would respond to both actinorhodin and intermediates; while the one expressing ActR\(^{T142W}\) would respond only to 3-ring intermediates.
Table 3.1 Cell-cell signaling between Donor and Recipient *S. coelicolor* strains

<table>
<thead>
<tr>
<th>Donor Strain (50%)</th>
<th>Recipient Strain (50%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M145</td>
<td>$^{(1)}$ΔactIΔactR/actR-P_{actAB}lux</td>
</tr>
<tr>
<td>ΔactVA-5</td>
<td>$^{(1)}$ΔactIΔactR/T142W-P_{actAB}lux</td>
</tr>
<tr>
<td>ΔactI</td>
<td>ΔactIΔactR/actR-P_{actAB}lux</td>
</tr>
</tbody>
</table>

$^{(1)}$Wild type or mutant actR gene is *in cis* constructed with $P_{actAB}$ directed luminescent reporter as indicated in Figure 2.9A.

In the absence of actinorhodin biosynthesis (ΔactI donor), *actAB* expression in neither wild type nor the T142W mutant recipient was activated, consistent with an early observation indicating an absolute requirement of actinorhodin pathway molecules for *actAB* induction (Figure 3.2). When a wild type donor was used (Figure 3.2A, top), induction of $P_{actAB}$ was observed following the actinorhodin production when the promoter was controlled by the wild type ActR, as expected. However, $P_{actAB}$ under control of the T142W mutant remained inactive (Figure 3.2A, bottom), suggesting that wild type *S. coelicolor* does not export pathway intermediates. When using the ΔactVA-5 mutant which is blocked at making 3-ring intermediates as the donor, induction of *actAB* promoter was observed in both types of recipients (Figure 3.2B) though 20-30 times weaker in intensity than that by the wild type donor. These experiments were performed in liquid culture, but similar results were found when repeated on solid medium (Figure 3.3).
Figure 3.2 Actinorhodin can act as an intercellular signal in wild type cells (liquid medium)

An actI ORF1-3 deletion mutant recipient strain bearing a fusion of P_{actAB} to luxCDABE was grown with (A) wild type strain M145 which can induce P_{actAB}-lux when it is under the control of wild type ActR (top) but not the T142W mutant (bottom), or (B) the intermediates producer ΔactVA-5 mutant which can induce P_{actAB} under the control of both wild type ActR (top) and the T142W mutant (bottom). A_{640}, indicative of actinorhodin production in shown with dashed lines and luminescence in the recipient strains, indicative of P_{actAB} activity, is shown with solid lines. The blue and green lines show the results of mixed cultures where M145 or an actVA-5 mutant acts as donor (A and B respectively). The red lines show the effects of when an actI deletion strain serves as donor strain.
Figure 3.3 Actinorhodin can act as an intercellular signal in wild type cells (solid medium).

The recipient strains in which $P_{actAB}$ is under control of wild type ActR (Left column) or T142W (Right column) were inoculated with donor strains (from top to bottom panel: M145, $\Delta actVA-5$ and $\Delta actI$) in 1:1 ratio on 96 well plates at a series of concentrations from 10 c.f.u. to $10^5$ c.f.u. (coded with cyan, purple, green, red, and blue colors) and read for $P_{actAB}$ directed luminescence. Similar to what indicated in Figure 3.2, M145 can induce $P_{actAB}$ only when it is controlled by WT ActR, while $\Delta actVA-5$ can induce $P_{actAB}$ regardless whether it is controlled by WT or mutant ActR, however, at lower intensity.

It is possible that most of the recipient cells which had $actAB$ under the control of the mutant ActR were killed by the actinorhodin produced by M145 late in the time course as they failed to respond the mature antibiotic (Figure 3.2A, bottom). On the other hand, we have been informed by collaborators (Koji Ichinose lab) that the 3-ring intermediate is subject to chemical modification and we have evidence from previous work that (S) DNPA turns colour with prolonged exposure to oxygen (Dr. Andrew Willems), all
suggesting that these molecules may not be as stable as actinorhodin when secreted outside the cell or may be inefficient to relieve the repression on \( P_{actAB} \) in extracellular environment\textsuperscript{155, 157}. Therefore, even though these intermediates can exhibit weak cell-cell signaling activity when “forced” to accumulate at higher level (such as in \( \Delta actVA-5 \) mutant, Figure 3.2B), they are either secreted in such a small amount that they rapidly turn into non-functional derivatives or they are not exported from wild type cells at all. We suggested that the reduced activity of the T142W-regulated \( lux \) reporters is due to the failure of actinorhodin-mediated intercellular signaling.

### 3.3.2 Intermediate triggered expression of \( P_{actAB} \) is not sustained throughout the culture

As a second means to address the cause of the discrepancy of \( P_{actAB} \) activities controlled by either wild type or T142W mutant ActR (Figure 2.8B and 2.9B), I constructed a set of transcriptional reporters based on enhanced green fluorescent protein (eGFP) to visualize \( actAB \) induction within colonies, in which \( P_{actAB} \) directed \( egfp \) gene is \textit{in cis} controlled by either wild type ActR or T142W mutant allele (similar to Figure 2.9A). We introduced the fusion constructs into \( actR \) deletion mutant as well as an \( actR \) \( actI \) double null mutant for negative control and the fluorescence was examined 5 hours and 24 hours after resuspension of overnight cultures in RG-2 medium.

\textit{S. coelicolor} grows as microcolonies of thousands of filamentous cells in liquid culture, with mycelium tubes expanding from the periphery of a condensed center mass (Figure 3.4). This mycelial mass is a complex multicellular cluster composed of both
early and late differentiated cells so that it is difficult to distinguish “individual” cells. I found that the cell cluster (both wild type and T142W mutant) glowed as a whole and no scattered lightening of cell patches was detected within the T142W cell cluster. Therefore, instead of studying the heterogeneity within each colony, we considered each cell cluster as a functional unit to display green fluorescence and revised “cell-cell communication” in submerged growth condition to “colony-colony communication” though the signaling integration within the cluster remains to be investigated.

At the early time point (5 hours after subculture, Figure 3.4 A and B), I observed a fluorescence signal that was dependent on the induction of P<sub>actAB-egfp</sub> by molecules from the actinorhodin biosynthetic pathway (compare fluorescence in actI<sup>+</sup> cells with lack thereof in actI<sup>-</sup> cells). Quantified fluorescence is shown in the scatter plots to the right. Cell clusters lacking actinorhodin biosynthesis generated a background fluorescence of 0-2 units, regardless of whether P<sub>actAB-egfp</sub> was under the control of the wild type ActR protein or the T142W mutant. In contrast, in the actI<sup>+</sup> cells, most clusters were significantly above this level. For example 79/84 cell clusters expressing the wild type ActR exhibited fluorescence above 2 units with maximum signals of ~10. A subset of the clusters (5/84) was at background fluorescence. In cells where P<sub>actAB-egfp</sub> was under the control of the T142W mutant, a majority of cell clusters (28/44) exhibited fluorescence > 2 units with maximum signals of ~9 units.

At the later time point (24 hour after subculture, Figure 3.4 C and D) when extracellular actinorhodin was detected, the phenotypes of the wild type ActR and T142W mutants were markedly different. The majority (32/44) of the T142W mutant
clusters were at background fluorescence with only 8 exhibiting signals of 2-3 units. All the wild type ActR cell clusters (50/50) exhibited greatly elevated fluorescence levels with maximum signals of 65 units.
Figure 3.4 Distribution of actAB induction among S. coelicolor colonies at early time points (A) and (B) and late time points (C) and (D). 

P_{actAB-egfp} fusions expressing wildtype ActR (A and C) or the T142W mutant (B and D) were introduced into an actR null mutant (actI+) or into an actR actI double null mutant (actI-) as indicated such that the reporter construct was the sole source of ActR protein. Fluorescence levels were quantified and expressed in arbitrary units. The green dots show the fluorescence levels in actI+ microcolonies while the olive coloured dots show the fluorescence levels in the actI- microcolonies. At early stage (A and B), P_{actAB} inductions were observed from a portion of both WT and T142W strains. At late stage (C and D), this activation was short-lived in T142W mutant, but was strengthened and widespread in the WT strain.

These data reproduce what we observed with the lux fusions and suggest that during the early stage of actinorhodin biosynthesis intermediates can act as intracellular signals. Given that the intermediates do not act as intercellular signals (Figure 3.2 and 3.3) in wild type cells, we presume that the intermediate-driven signaling is intracellular and serves to trigger the expression of actAB promoter at sufficient levels for wild type yields and export of actinorhodin. Once the actinorhodin production is established, the mature antibiotic could act as an intercellular signal to trigger the export genes throughout the culture at a sustained high level.

3.3.3 The resultant death in T142W that fails to sense intercellular signals

As the strain bearing the T142W mutation produces actinorhodin in a manner that is indistinguishable from the wild type, we wondered whether there was a phenotype price for failing to respond to the mature product actinorhodin: are they more vulnerable than the wild type cells when exposed to actinorhodin in the late course of cell culture?

Due to the filamentous morphology and massive clustering growth of submerged Streptomyces, it is not feasible to assess the number of viable cells by plating cells on a
suitable surface medium and counting the resulting colonies. Therefore, we applied a viability stain assay to analyze the death processes of both wild type M145 and in situ replaced T142W mutant under the same cultivation condition as that used in previous luminescent and fluorescent tests (Figure 3.5). Cells were harvested from Day 1 (late exponential/vegetative phase), Day 3 (stationary phase) and Day 8 (death phase) after inoculation and stained by SYTO 9 green fluorescent nucleic acid stain plus propidium iodide (PI) red fluorescent stain (v:v=1:1). SYTO 9 labels all the cells, while PI only enters cells with damaged membranes and due to its higher affinity to nucleic acids causes a reduction in SYTO 9 green. The green and red pattern would be monitored using fluorescent microscopy and provides an indication of the distribution of live (green) and dead (red) cells within the colony.

Similar to the previous fluorescent detections, submerged M145 and T142W cells aggregated as mycelial clusters with fuzzy periphery and a “core” area. Using the live/dead staining, the “red” core, consisting of mostly the dead cells, is surrounded by a “green” ring that delineats live cells on the outside (Figure 3.5A). The dying cells stayed part of the mass to provide mechanical and possibly nutrient supports for peripheral live cells. During early (Day1) and mid (Day3) stages of actinorhodin production, there was no observable difference between the M145 and T142W strains: both gave rise to microcolonies with a core of dead/dying cells surrounded by a ring of live cells (Figure 3.5A). However, in late stage of culture (Day 8), in marked contrast to the wild type microcolonies which contained this peripheral ring of viable cells throughout the culture, the T142W mutant exhibited widespread cell death (Figure 3.5A). Although consistent
with previous finding (Figure 2.8 and 2.9), the actinorhodin yields of T142W is not distinct from that of the wild type till the mid-stage of actinorhodin production (Figure 3.5B), actinorhodin yields by T142W mutant is only one third of that by the wild type in late culture (Figure 3.5B) which is likely due to the larger proportion of dead colonies.

Figure 3.5 Viability assays for wild type M145 and T142W mutant
(A) Comparison of cell growth phenotypes were made between M145 (left) and a congeneric T142W mutant (right) on day1, day3 and day8 following inoculation. Cells that stain green are impermeable to PI while those that stain red are permeable and therefore have damaged cell walls. The day1 and day3 M145 and T142W microcolonies are indistinguishable by this assay, consisting of a red fluorescent core of dead cells and an outer ring of green viable cells. On day 8, the M145 microcolonies again consisted of a core of dead cells surrounded by viable cells, while microcolonies of the T142W mutant were smaller and stained red throughout, indicating a death wiping out the mutant culture.

(B) Actinorhodin production, indicated by $A_{640}$, was tested in M145 (blue) and T142W (red). There is no significant difference in actinorhodin yields on day1 and day3, consistent with results shown in chapter II. However, actinorhodin productions were reduced in late culture of both strains (day8) and the yield by T142W was only one thirds of that by M145.

These observations are consistent with the eGFP experiments (Figure 3.4) which showed both wild type and T142W mutant microcolonies exhibited low level $P_{actAB}$ activity in younger cultures but the sustained, high level expression only took place in the older culture of wild type cells. Therefore, while intermediate-driven intracellular signaling activates $actAB$ for export from actinorhodin producing cells early during the cell culture, actinorhodin-mediated intercellular signaling is essential for sustained, microcolony-wide $actAB$-mediated resistance.

3.4 Conclusion and discussion

I have shown previously that the intermediates can trigger export from the producing cells. The newer finding in this chapter demonstrates that the absence of the intercellular signalling step mediated by actinorhodin could be detrimental for the organism. It is also interesting to notice that the wide spread death of T142W was not immediate but lagged behind the peak of actinorhodin production. This phenomenon suggested that other resistance strategies (e.g. other efflux pumps) might exist to complement the hindered
actAB expression to some extent and temporarily protect the cell. Further more, the widespread death of T142W mutant also indicates that actinorhodin molecules are antibiotics. However, having been characterized on its biosynthetic pathway for over three decades, the action mode of actinorhodin is still quite blurry. Definition on its “moderately weak antibiosis” mostly came from early reports of its inhibitory effects on *Staphylococcus aureus* (MIC$_{100}$ ≈ 25 µg). Recently, preliminary experiments performed by Stefanie Mak from our lab revealed that actinorhodin may possess unexpected potent inhibition against the natural competitors of *Streptomyces* such as *Bacillus subtilis* as well as some other pathogenic bacteria, which once again confirms the importance of advanced activation of export and resistance mechanisms in actinorhodin producing cells.

### 3.5 Methods and Materials

#### Table 3.2 Bacterial strains and plasmids used in this chapter

<table>
<thead>
<tr>
<th>Strain/plasmid/cosmid</th>
<th>Description/function</th>
<th>Source/Creator</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. coelicolor</em> M145</td>
<td>Wild type host strain, SCP1’, SCP2’</td>
<td>John Innes Centre</td>
</tr>
<tr>
<td><em>S. coelicolor</em> actI::specR</td>
<td>M145, actI-orf1/2/3 replaced by spec$^{R}$ cassette</td>
<td>Ye Xu</td>
</tr>
<tr>
<td><em>S. coelicolor</em> actI::kanR</td>
<td>M145, actI-orf1/2/3 replaced by kan$^{R}$ cassette</td>
<td>Kapil Tahlan</td>
</tr>
<tr>
<td><em>S. coelicolor</em> actVA-5::apraR</td>
<td>M145, actVA-orf5 replaced by apra$^{R}$ cassette</td>
<td>Ye Xu</td>
</tr>
<tr>
<td><em>S. coelicolor</em> ΔactVA-5</td>
<td>M145, ΔactVA-5 deletion mutant</td>
<td>Ye Xu</td>
</tr>
<tr>
<td><em>S. coelicolor</em> ΔactIΔactR</td>
<td>M145, ΔactIΔactR deletion mutant</td>
<td>Ye Xu</td>
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<tr>
<td><em>S. coelicolor</em> ΔactR</td>
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<td>Ye Xu</td>
</tr>
<tr>
<td><em>S. coelicolor</em> ΔactI</td>
<td>M145, ΔactI deletion mutant</td>
<td>Ye Xu</td>
</tr>
<tr>
<td><em>S. coelicolor</em> T142W</td>
<td>M145, actR$^{T142W}$ mutant</td>
<td>Ye Xu</td>
</tr>
<tr>
<td><em>E. coli</em> XL1-Blue</td>
<td>Host strain for DNA cloning <em>recA1, endA1, gyrA96, hsdR17, supE4, relA1</em> lac[F’ proAB lacI$q^Q$ ΔM15Tn10(Tet$^r$)]</td>
<td>Stratgene</td>
</tr>
<tr>
<td><em>E. coli</em> ET12567/pUZ8002</td>
<td>Host strain for DNA conjugation, containing the non-transmissible <em>oriT</em> mobilizing plasmid pUZ8002 <em>dam13::Tn9, dcm-6, hsdM, hsdR, recF143</em></td>
<td>Kieser <em>et al.</em> (2000)</td>
</tr>
</tbody>
</table>
4.3.1 Bacterial strains and measurement conditions

Strains and plasmids used in this study are described in Table 3.3. The growth conditions and routine molecular genetic procedures for *Streptomyces* and *E. coli* were described in Experimental procedures of Chapter II.
3.5.2 Procedures for DNA manipulation

Standard procedures were used for plasmid/chromosomal DNA/cosmid isolation, subcloning and analysis (Sambrook et al. 1989; Kieser et al., 2000). Digestions and ligations were performed using restriction enzymes and T4 DNA ligase from NEB. All subclonings were subjected to end-to-end sequence confirmation.

3.5.3 Cell-cell signaling assays by mixing culture

For liquid mix culture, the three donor strains (M145, ΔactVA-5 and ΔactI) and the two recipient/reporter strains (ΔactRΔactI/pWT +IGR+lux and ΔactRactI/pT142W+IGR+lux) were inoculated into 40ml R2 liquid medium (without yeast extract) at equal concentration of 0.25 x10^6 spores/ml and grown at 30℃ with shaking at 200 rpm for 5 days. The luminescence readings and actinorhodin assays were taken twice every day, normalized to the wet weight of cell pellet and plotted as a function of time as described in Chapter II.

For the solid mix culture, the same donor and recipient strains were mixed in pair at a series of concentrations (10^5, 10^4, 10^3, 10^2 and 10 c.f.u.) and grown on 96 well MS agar plate for 3 days. Luminescent readings were taken twice every day by VICTOR™ X Multilabel Plate Readers (Perkin Elmer).

3.5.4 eGFP fluorescent assays

To generate a transcriptional green fluorescent reporter that is directed by the actAB promoter and mediated by either wild type ActR or mutated ActR, the gene fragment of actR/actR_{T142W} along with actR.actAB intergenic region (IGR) was cut from actR-
1. \( \text{P}_{\text{actAB}} \text{lux} / \text{T142W-} \text{P}_{\text{actAB}} \text{eGFP} \) (Experimental procedures, Chapter II) by BamHI and KpnI, and ligated into the same restriction sites of pIJ8660 to give pWT+IGR+eGFP/pT142W+IGR+eGFP. The generated plasmids were introduced into \( \Delta \text{actR} \) mutant and \( \Delta \text{actR}\Delta \text{actI} \) mutant by conjugation, to give four \textit{Streptomyces} reporter strains: \( \Delta \text{actR}/\text{actR-} \text{P}_{\text{actAB}} \text{eGFP} \), \( \Delta \text{actR}/\text{T142W-} \text{P}_{\text{actAB}} \text{eGFP} \), \( \Delta \text{actR}\Delta \text{actI}/\text{actR-} \text{P}_{\text{actAB}} \text{eGFP} \) and \( \Delta \text{actR}\Delta \text{actI}/\text{T142W-} \text{P}_{\text{actAB}} \text{eGFP} \). All four strains were inoculated in R2YE liquid medium at \( 0.5 \times 10^6 \) spores/ml and grown at \( 30^\circ \text{C} \) with shaking at 200 rpm until \( \text{OD}_{450} \) reached approximately 0.1. Cells were recovered by centrifugation at 3000 rpm, washed once with 0.85% saline and resuspended in RG-2 medium and incubated at \( 30^\circ \text{C} \) with shaking at 200 rpm for another two days. Fluorescence microscopy was performed on a Zeiss Axio Imager M2 microscope (Axiovision Rel. 4.8, x10 objective) to detect mycelium grown at 0 hour, 5 hours, 24 hours after subculture in RG-2 liquid medium. To compensate for the auto-fluorescence of \textit{S.coelicolor}, the two \( \Delta \text{actR}\Delta \text{actI} \) mutant hosts that were deficient in making signaling molecules were used as negative controls to lower the visualization of fluorescence background, and the same parameters were applied to \( \Delta \text{actR} \) strains that host the same type of eGFP reporter. All images were processed by Photoshop CS5.1 uniformly. The fluorescence intensity of each cell cluster was normalized and quantified by Image J, and plotted by comparing density distributions between \( \Delta \text{actR}\Delta \text{actI} \) control and \( \Delta \text{actR} \) reporter.
3.5.5 The viability assay for M145 and T142W

M145 and T142W were inoculated into R2YE medium, grown to exponential phase and subcultured into RG-2 medium as described before (Method and Material, Chapter II). One millilitre of culture was taken on Day1, Day3 and Day8 after subculture. Samples were centrifuged for 5 min at 12,000 rpm, washed twice with 0.85% saline and resuspended in 500 µl water. Each resuspension was mixed with 3 µl of SYTO 9 green plus propidium iodide (PI) red fluorescent nucleic acid stains (Light Bacterial Viability Kit, Invitrogen, L13152, prepared 1:1 v/v, as recommended by the manufacturer). SYTO 9 labels all the cells, with intact or damaged membranes, while PI only enters the damaged cells, causing a reduction in the SYTO 9 stain. The cell suspension and the stain mixture were mixed by pipetting or inverting the tubes and left to stand at room temperature for 20 min in the dark. 10 µl of the suspension was deposited onto a clean slide and examined under Zeiss Axio Imager M2 microscope (Axiovision Rel. 4.8, x10 objective). Exposure time for red channel and green channel was 10ms. Images from green and red channels were merged and processed by Photoshop CS5.1.
Chapter IV

Conclusions and remaining questions arising from my work
Although the participation of precursor molecules in initiation of resistance genes was suggested in early studies, these ideas were speculative attempts to explain the reason that resistance genes were cotranscribed with structural genes (e.g. Figure 1.2 oxytetracycline) or under the control of the pathway specific activator (e.g. Figure 1.4 spiramycin). Some examples have specified this coordination at a functional level in which the resistance gene products are enzymes that modify the pathway intermediates (e.g. Figure 1.7 streptomycin) or the final products (e.g. Figure 1.6 oleandomycin) during the biosynthetic process. However, even though the interaction between intermediate molecules and the regulator of cognate resistance genes was characterized \textit{in vitro} in a few cases (e.g. Figure 1.12 doxorubicin), it was still controversial whether the products from antibiotic biosynthetic pathway are relevant to the activation of resistance mechanisms in producers. By creating a mutant responding to only the 3-ring intermediate and using a combination of transcriptional reporters, I demonstrated that the precursors and the mature actinorhodin molecules provide different functions in the control of actinorhodin export/resistance: The precursor metabolites initiate expression of the \textit{actAB} genes. In this way, the producer cells ensure that the pumps are available to drive efficient actinorhodin production and to protect themselves from self-killing before the mature antibiotic accumulates to detrimental concentrations. Apparently however, this initial burst of \textit{actAB} expression is not sustained and drops off with time. A second \textit{actAB} activation step is driven by mature, secreted actinorhodin to sustain expression of these genes across the culture and to protect \textit{S. coelicolor} from the antimicrobial effects of the actinorhodin as it accumulates later on during cell culture.
My work presented in this thesis is the first concrete in vivo evidence for the activation of export/resistance via pathway intermediates from a secondary metabolite gene cluster. Furthermore, it encompasses a significant advancement in understanding the link between antibiotic resistance and biosynthesis, which is important for our efforts to manipulate secondary metabolism. For example, many secondary metabolites are produced at low levels: my work suggests that manipulating export might be one way to enhance yields of these molecules. More importantly, the knowledge we gained from understanding the antibiotic biosynthesis and cognate resistance mechanism in S. coelicolor could be expanded to other producers of bio-active molecules and will be relevant to our ability to control the emergence of antibiotic-resistance among pathogenic bacteria. My work represents only a small fraction of the Nodwell lab’s efforts on solving the problem of antibiotic resistance and a lot of intriguing subjects have arisen from this project. In this last chapter, I present some of them and hope my speculation and preliminary studies on these interesting topics will lead to more investigations in diverse areas.

4.1 The role antibiotic exporter plays in antibiotic biosynthesis

First, what is the role of the putative export proteins during biosynthesis and is export a general means of enhancing secondary metabolite yields? As efflux pumps are predicted to reduce the intracellular accumulation of the drugs, inactivation of these exporters might result in severe toxicity to the producing organism in some cases. For example, inactivation of the cmrAB operon which encodes a chromomycin ABC transporter system
was not achieved in *S. griseus subsp. griseus*, suggesting that expression of these genes is essential to the chromomycin producer. On the other hand, these export proteins seemed not merely involved in drug transport but could mediate drug production in some way as well. It is not a rare case to find that the export genes (as well as other type of resistance genes) are present in vicinity with genes encoding transcriptional regulators. Expression of the *cmrAB* encoded chromomycin exporter is believed to be repressed by CmmRII. Disruption in *cmmRII* led to an earlier (≈ half day earlier) and a higher yield (≈ 70% higher) of chromomycin in this mutant, similar to what we observed in our *actR* deletion mutant. It is possible that initiation of export relieves the end product inhibition and ensures the biosynthesis goes efficiently. Or these transmembrane proteins could play a more active role to support the biosynthetic machinery. For example, they might serve as the platform and assemble the core biosynthetic enzymes as an integrated complex to facilitate processing of the intermediate molecules. And the timing for this assembly might be controlled by regulatory proteins like ActR that sense the biosynthetic progress by directly binding to the precursor molecules.

### 4.2 Evidence of cooperation between antibiotic biosynthesis and export/resistance in other antibiotic producers

Another interesting question is: is the mechanism of “priming and spreading the export and resistance” by intermediates and secreted secondary metabolites employed by other producers of bioactive molecules, particularly by those that generate molecules with deleterious effects on the producing cells? I have initiated other projects to learn about the
relationship between secondary metabolism and export/resistance in \textit{S. coelicolor}. For example, outside the biosynthetic gene cluster of undecylprodigiosin, another pair of tetR-tetA like genes, \textit{SCO4008-SCO4007}, was found related to the yield of this red pigmented antibiotic. Knocking out the repressor gene \textit{SCO4008} increased not only the “red” production by 30\%-40\%, but surprisingly also promoted expression of some critical structural genes (Information and preliminary data about undecylprodigiosin biosynthesis and export can be found in Appendix 3). This effect is not well understood at all at the moment and the role of this tetR-tetA pair in red biosynthesis and export remains to be further characterized.

4.3 ActR mediated ActA/ActB like export and resistance in other microorganisms

We are also interested in the specificity of the ActR mediated ActA and ActB export/resistance. Could the ligand specificity of regulator differ from that of its controlled drug transporter, or do they coevolve to adapt to the same groups of substrates? For example, in contrast to the aforementioned TetR-TetA model which is highly specific for tetracycline related molecules, the QacR controlled QacA transporter confers resistance to a broad range of antimicrobial agents in \textit{Staphylococcus aureus} and the QacR protein interacts directly with structurally dissimilar compounds that are substrates of the QacA multidrug efflux pump\textsuperscript{162-164}. As indicated by previous experiments\textsuperscript{58}, the ligand binding cavity of ActR could accommodate a wider variety of molecules than we originally expected\textsuperscript{115}. All were synthetic molecules containing the anthraquinone core and many of them could release ActR from the DNA operator both \textit{in vitro} and \textit{in vivo}\textsuperscript{115}.
Therefore, it would be interesting to know whether ActR respond to other natural antimicrobial compounds and the ActA/ActB confer resistance to these same molecules.

Another perspective to view these questions is whether any actinorhodin non-producing organisms adopted or developed an ActR-ActA/ActB like resistance mechanism such that they could respond to actinorhodin and activate resistance in a similar way to that in *S. coelicolor*. By performing a similarity search of the ActR protein sequence using BLAST in the annotated *Actinomyces* genome data base (Actinomycetales group proteins, Broad Institute) (Table 4.1), I surveyed the top 100 hits from 18 out of 19 species, all belonging to the TetR family regulator (TFR), as well as the products encoded by their neighbouring genes. It revealed that 41 of them are encoded adjacent or close to at least one putative transporters (e.g. ABC transporter, MFS transporter) (Table 4.1), some of which were annotated as multidrug resistance efflux pumps (e.g. SSGG_05839 in *S. roseosporus* NRRL 15998) or resistance proteins to specific antibiotics (e.g. SSCG_03624 in *S. clavuligerus* ATCC 27064 to puromycin, SSAG_00775 in *S.sp* Mg1 to bleomycin). Interestingly, there are three MFS export proteins with protein sequence similarity to ActA from three different organisms (highlighted in Table 4.1): KUTG_02528 in *Kutzneria sp*. 744, SSPG_02606 in *S. lividans* TK24, and SSTG_05028 in *S. sp* E14. As *S. lividans* TK24 is known to produce actinorhodin, it is not surprising to find a copy of ActR-ActA (SSPG_02605-SSPG_02606) as well as other actinorhodin biosynthetic enzymes (e.g. *actVA-4*) encoded as a cluster in its genome. The potential of actinorhodin production in *Kutzneria sp*. 744 and *S. sp* E14 is not known, and genes encoding enzymes with diverse similarity to some structural or tailoring enzymes.
of actinorhodin biosynthesis were found scattered within their genomes (e.g. 
*KUTG_03884* to *actI-orf1, KUTG_02091* to *actVI-orf1*). However, as both *Kutzneria* and 
*Streptomyces* are promising bacteria in polyketide synthesis, it is common to find 
orthologues of polyketide synthase and tailoring enzymes encoded in their genome. No 
actinorhodin was detected from the *Kutzneria* cultured under our lab condition (Dr. Sheila 
Elardo) and there is no literature indicating that *Kutzneria sp.* 744 or *S. sp* E14 yields this 
blue antibiotic. Furthermore, the TFR *KUTG_02529* is phylogenetically proximal to 
*ActR* (Dr. Leslie Cuthbertson) although its specificity for actinorhodin requires further 
investigation. Therefore, actinorhodin mediated export/resistance mechanism might exist 
in non-producers and bacteria possessing an intact *ActR-ActA/ActB* like system might 
confer resistance to actinorhodin regardless of its capability to produce this molecule.

In addition, the remaining 59 *ActR* homologues are adjacent to genes encoding 
hypothetical proteins, transcriptional regulators (including *TetR* family regulator less 
similar to *ActR*), oxidoreductases, monooxygenases, methyltransferases, *etc.* One cannot 
exclude the possibility that some of them are resistance proteins other than the efflux 
pump. In fact, it is not rare that antibiotic non-producing isolates acquire resistance 
mechanisms that differ from those employed by the producing organism. Therefore, we 
could expect a variety of actinorhodin responsive resistance mechanisms in actinorhodin 
non-producers and resistance other than efflux could also suggest the mode of action of 
actinorhodin, but we need to testify the actinorhodin specificity of these TFRs and the 
actinorhodin resistance conferred by their paired proteins in first place.
<table>
<thead>
<tr>
<th>Species (Hits)</th>
<th>Gene locus</th>
<th>Residue Identity</th>
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<td>SSHG_03053 Transmembrane efflux pumps</td>
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<td><em>S. sviceus ATCC 29083 (1/4)</em></td>
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<td><strong>KUTG_02528</strong> Actinorhodin transporter?</td>
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<td><em>S. ghanaensis ATCC 14672 (3/3)</em></td>
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4.4 Influence on *S. coelicolor* development by actinorhodin

Last but not least, although actinorhodin production is not essential for cell growth, it might have subtle effects on global features such as morphological development in *S. coelicolor*. I conducted a competition assay by co-culturing actinorhodin producers with non-producers. As shown by Figure 4.1, the producer of actinorhodin (M145) or the producer of the 3-ring intermediates (*ΔactVA*-5 mutant) was mixed with non-producer, of which the polyketide synthase genes (*actI-orf1/2/3*) were replaced with a kanamycin resistance marker (*actI:: Kan*R*), and both strains were plated on solid agar medium. The starting ratio of inoculum was either 50%: 50% (left panel), or 5% producer: 95% non-producer (right panel). The mixed culture was harvested, selected by kanamycin and the survival ratio of producer: non-producer was determined by counting colonies once weekly for a month. Surprisingly, the wild type M145 gradually overwhelmed the *actI:: Kan*R* and was superior in number to its non-producer opponent in a month (Figure 4.1A). Starting with equal inoculums, the proportion of M145 increased from around 50% to 86% and the same tendency was observed when starting with a smaller inoculum of M145 (5%) which ended up as the majority (59%) of the two strains after four weeks. The advantage
of ΔactVA-5 mutant over actI: KanR was not obvious (Figure 4.1B) and was not included in following studies. The growth imbalance between M145 and actI deletion mutant is surprising because the non-producer possesses the ActR mediated efflux pumps (ActA and ActB) which would ensure protection in response to the antibiotic.

Figure 4.1 Growth competition between actinorhodin producer and non-producer
Actinorhodin producer (M145 in blue or ΔactVA-5 in orange) is mixed cultured with non-producer (actI: KanR mutant in red) with inoculation ratio of either 50%: 50% (left) or 5% producer: 95% non-producer (right). (A) M145 Vs. actI: KanR mutant. (B) ΔactVA-5 mutant vs. actI: KanR mutant. While M145 gradually outnumbered actI mutant, the superiority of ΔactVA-5 over actI mutant was not evident.

One possible explanation for this observation is that the actI: KanR mutant, due to its lost capacity to produce actinorhodin, is deficient in initiation of actAB by endogenous intermediates and might be impaired in expression of morphological genes (e.g. sporulation genes) before the export/resistance is activated by exogenous actinorhodin
(from M145), resulting in a developmental disparity between the two strains. To test if expression of any sporulation genes was influenced by actinorhodin, I grew an \( \Delta \text{actI} \) null mutant bearing fusions of \( P_{\text{whiA}} \), \( P_{\text{whiE}} \) or \( P_{\text{actAB}} \) to the luminescent reporter by itself (Figure 4.2 red line) or in a mixed culture with M145 (Figure 4.2 blue line). \( P_{\text{whiA}} \) in individually growing \( \Delta \text{actI} \) was activated around 40 hours after inoculation, reached the peak activity around 100 hour and went down. \( P_{\text{whiE}} \) in \( \Delta \text{actI} \) was activated around 30 hour and expressed consistently during the sporulation course. As we expected, induction of \( P_{\text{actAB}} \) is actinorhodin dependent and was only detected when growing with M145. However, it was not turned on until \(~50\) hours and was off around \( 100\) hours. Relevant to this late induction of \( P_{\text{actAB}} \), activation of \( P_{\text{whiA}} \) was delayed for at least 20 hours and that of \( P_{\text{whiE}} \) for over 50 hours in \( \Delta \text{actI} \) mutant when mixed cultured with M145. These preliminary results suggested that first, actinorhodin could influence sporulation process in \( S. \text{coelicolor} \). The mechanism is not understood at the moment but more genes from sporulation pathways (e.g. \( \text{whiG}, \text{whiH}, \text{whiD}, \text{etc.}^{167} \)) could be tested in \( \Delta \text{actI} \) mutant and compared for their expressions in the presence/absence of actinorhodin; second, although signals that trigger actinorhodin biosynthesis in substrate mycelium have not been characterized, it seems to be important that co-habited cells produce actinorhodin all in step so as to synchronize the resistance initiation. Cells that lag behind or deficient (e.g. \( \Delta \text{actI} \)) in turning on the actinorhodin biosynthetic machinery could be temporarily attacked by exogenous actinorhodin until the export/resistance is induced to release the toxicity.
Figure 4.2 *whiA* and *whiE* expression in actinorhodin non-producer $\Delta actI$ is affected by actinorhodin

$\Delta actI$ bearing $P_{\text{whiA}}$-lux, $P_{\text{whiE}}$-lux or $P_{\text{actAB}}$-lux were co-cultured with M145 (blue line) or by itself (red line). $\Delta actI$ bearing empty vector was used as negative control and also grown with M145 (green line) or by itself (purple line). Inductions of $P_{\text{whiA}}$ and $P_{\text{whiE}}$ were both detected in $\Delta actI$ growing by itself but much delayed when growing with M145, possibly due to a late induction of $P_{\text{actAB}}$ in $\Delta actI$ by co-cultured M145. $P_{\text{actAB}}$ activation is actinorhodin dependent and was not detected in $\Delta actI$ growing by itself.

Another possibility that caused the growth imbalance between M145 and *actI* mutant could be the intrinsic slowness of sporulation in the actinorhodin non-producer, as a compromised sporulation in individually growing actinorhodin non-producers (e.g. $\Delta actI$) was observed when compared with that in wild type M145 using scanning electron microscopy (Figure 4.3).
Figure 4.3 Morphological development of M145, ΔactVA-5 and ΔactI under scanning electron microscope

All types of strains developed into aerial hyphae with regular septums. Spore chains of M145 (Day3, left) matured earlier than that of other two strains.

Although neither PwhiA nor PwhiE activity in ΔactI was found significantly different from that in M145, expression of other sporulation genes might be delayed in non-producer and requires further investigations. Moreover, work from the Newman lab and following studies from other two labs suggested a relationship between actinorhodin and a response regulator of oxidative stress which might modulate life cycle in non-enteric bacteria.  

Transcriptional factor, SoxR, is a well characterized stress response regulator in *E. coli* or *Salmonella enterica*. In these bacteria, it activates a downstream transcriptional regulator SoxS which controls genes involved in superoxide resistance or detoxification. However, bioinformatics and gene expression studies
revealed that occurrence of SoxS was restricted to the family *Enterobacteriaceae* and SoxR might play alternative roles in non-enteric bacteria (e.g. *Pseudomonas* and *Streptomyces*) such as responding to redox-active molecules and mediating other aspects of microbial behaviour (e.g. iron acquisition, energy generation and biofilm formation). In *S. coelicolor*, the SoxR homologue (SCO1697) regulates five genes encoding a putative ABC transporter (SCO7008), two oxidoreductases similar to ActVA-Orf2/Orf4 (SCO2478 and SCO4266), a monoxygenase similar to ActVA-Orf6 (SCO1909), and a possible NAD-dependent epimerase/dehydratase (SCO1178) respectively. Expression of these genes requires the intact [2Fe-2S] cluster in SoxR and depends on actinorhodin production. Further more, the *soxR* null mutant was not compromised in resistance against oxidizing agents but was slow in aerial hyphae formation with reduced sporulation and decreased pigment (actinorhodin and undecylprodigiosin) production. The researchers suggested that SoxR might be another signal responder of actinorhodin like redox-active agents and activate expression of a series of downstream targets to initiate protections against these molecules or to ensure optimal differentiation or production of secondary metabolites.

To understand the relationship between actinorhodin biosynthesis and the SoxR mediated signal transduction in *S. coelicolor*, at least two questions remain to be characterized. First, how does actinorhodin regulate SoxR activity, via direct interaction, or more likely via the [2Fe-2S] cluster in SoxR which may sense the superoxide generated by actinorhodin or its precursors? If the latter is true, this would be another hint.
for actinorhodin action mode. Second, what is the biological relevance of SoxR activity? On the basis of the identities of the genes in SoxR regulon, the most obvious potential is the protection from endogenously produced antibiotic. I speculated that the ABC transporter encoded by SCO7008 might play a compensative role in the absence of the major actinorhodin export (ActA/ActB). The other four members of SoxR regulon may potentially mediate actinorhodin production or detoxification, but alternative functions cannot be excluded. Although there are a lot of debates, increasing comprehension of antibiotic molecules which are typically viewed as waste products or arms in bio-warfare suggested their association with morphological differentiation in producing organisms\textsuperscript{175}. Therefore, further investigation on the regulatory cascade of actinorhodin→SoxR regulon→physiology/secondary metabolism would help envision the physiological and ecological roles of secondary metabolites in bacterial community.

Taken together, all these questions are still highly speculative and very interesting. Further research will provide comprehensions on roles that export/resistance system plays in the cognate antibiotic biosynthesis and help understand transfer and evolution of these two events among producing and non-producing organisms. Specifically, these questions are also good starts to unravel many intriguing aspects of the blue pigmented antibiotic such as its mode of action and target sites, and lead us to unknown crosslinks between pathways of secondary metabolites and cell differentiation.

\textbf{4.5 Methods and materials}

\textbf{Table 4.2 Bacterial strains and plasmids used in this chapter}
<table>
<thead>
<tr>
<th>Strain/plasmid/cosmid</th>
<th>Description/function</th>
<th>Source/Creator</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. coelicolor M145</strong></td>
<td>Wild type host strain, SCP1&lt;sup&gt;-&lt;/sup&gt;, SCP2&lt;sup&gt;-&lt;/sup&gt;</td>
<td>John Innes Centre</td>
</tr>
<tr>
<td><strong>S. coelicolor actI::kan&lt;sup&gt;R&lt;/sup&gt;</strong></td>
<td>M145, actI-orfI/2/3 replaced by kan&lt;sup&gt;R&lt;/sup&gt; cassette</td>
<td>Kapil Tahlan</td>
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<td><strong>S. coelicolor ΔactVA-5</strong></td>
<td>M145, ΔactVA-5 deletion mutant</td>
<td>Ye Xu</td>
</tr>
<tr>
<td><strong>S. coelicolor ΔactI</strong></td>
<td>M145, ΔactI deletion mutant</td>
<td>Ye Xu</td>
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<td><strong>E. coli ET12567/pUZ8002</strong></td>
<td>Host strain for DNA conjugation, containing the non-transmissible oriT mobilizing plasmid pUZ8002&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Kieser et al. (2000)</td>
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<tr>
<td><strong>E. coli DH5α</strong></td>
<td>Host strain for DNA cloning</td>
<td>Stratagene</td>
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<td><strong>pMU1</strong></td>
<td>Promoterless luxCDABE operon, flanked by transcriptional terminators, preceded by in-frame stop codons and ribosome binding sites, aac(3)IV&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Craney et al. (2007)</td>
</tr>
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<td><strong>pMU1-actABpLux</strong></td>
<td>pMU1, lux reporter directed by actAB promoter (apr&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Ye Xu</td>
</tr>
<tr>
<td><strong>pMU1-flux</strong></td>
<td>pMU1 background with inverted luxCDABE operon preceded by in-frame stop codons, ribosome binding sites and transcriptional terminator. The downstream transcriptional terminator was replaced by hairpin loops.</td>
<td>Craney et al.</td>
</tr>
<tr>
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<td>pMU1-lux, lux reporter directed by whiE promoter 1(apr&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Ye Xu</td>
</tr>
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<td>pMU1-lux, lux reporter directed by whiA promoter (apr&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Arryn Craney</td>
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<td><strong>pUZ8002</strong></td>
<td>Expression of protein for DNA conjugation in trans, kan</td>
<td>Kieser et al. (2000)</td>
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</table>

1 *amp<sup>R</sup>, ampicillin resistant; apr<sup>R</sup>, apramycin resistant; cam<sup>R</sup>, chloramphenicol resistant; kan<sup>R</sup>, kanamycin resistant; spc<sup>R</sup>, spectinomycin resistant

*1*
Table 4.3 Oligonucleotide primers used in this chapter

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<th>Name</th>
<th>Sequence (5’→3’)</th>
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<td>ACTCGTTGGATCCCATGATCCTCCGTTG</td>
<td>Forward primer for <em>whiEp1</em> promoter amplification, including BamH1 site</td>
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<td><em>whiEp1R</em></td>
<td>GCATCCCGTGACGTATAGCGACGAG</td>
<td>Reverse primer for <em>whiEp1</em> promoter amplification, including KpnI site</td>
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4.5.1 Bacterial strains and measurement conditions

Strains and plasmids used in this study are described in Table 4.2. The growth conditions and routine molecular genetic procedures for *Streptomyces* and *E. coli* were described in Experimental procedures of Chapter II.

4.5.2 Procedures for DNA manipulation

Oligonucleotides used in this study are listed in Table 4.3. Standard procedures were used for plasmid/chromosomal DNA/cosmid isolation, subcloning and analysis (Sambrook et al. 1989; Kieser et al., 2000). Digestions and ligations were performed using restriction enzymes and T4 DNA ligase from NEB. All subclonings were subjected to end-to-end sequence confirmation.

4.5.3 Cell competition assay

Strain A of actinorhodin and related molecule producers (M145 and ΔactVA-5) were paired with strain B which had actinorhodin biosynthetic gene replaced by apramycin or kanamycin resistance marker (*actI::Kan<sup>R</sup>* and *actVA-5:: Apra<sup>R</sup>*). The three pairs (M145||
actI::Kan\(^R\), M145||actVA-5::Apra\(^R\), and actVA-5::Apra\(^R\) ||actI::Kan\(^R\)) were plated onto MS agar plate starting with either equal amount of strain A and B, or 5% strain A and 95% strain B. The mixed spores were harvested after one week and 1 ml dilution (~ 2x \(10^2\) spores/ml) from the spore stock was plated on MS agar plate with relevant antibiotics to select survived strain B, or plate without antibiotics for counting total number of strain A and B. The survival ratio= (number of strain B colonies)/ (number of total colonies − number of strain B colonies). The rest of the mixed spores was plated on MS agar and grown for another week. The same selection and calculation procedures were carried out for four weeks. The survival ratio was determined for each week and the proportion of strain A and B was plotted as stacked column by excel.

4.5.4 Construction of luminescent reporter regulated by promoters of whiE

Oligonucleotide primers listed in Table 4.2 were used to amplify promoters that consist of the whole inter-genetic region by PCR from M145 chromosomal DNA. The fragment was ligated into BamHI and KpnI sites of pMU1-lux (for whiE genes), giving rise to luminescent reporter directed by these promoters (e.g. pMU1-whiEp\(_{\text{lux}}\)). This integrating plasmid was introduced into wild type S. coelicolor strain M145 or \(\Delta\)actI mutants by conjugation. Exconjugants were selected by apramycin (50 \(\mu\)g/ml) and nalidixic acid (25 \(\mu\)g/ml), and restreaked on R2YE agar/apramycin (50 \(\mu\)g/ml) for making spore stocks. For luminescent tests, equal amount of S. coelicolor (5x \(10^5\) spores) were inoculated on 96 well plates of MS or R2YE agar medium and read every day for 4-5 days by VICTOR\textsuperscript{TM} X Multilabel Plate Readers (Perkin Elmer).
4.5.5 Scanning electron microscopy

Wild type M145, ΔactVA-5 and ΔactI strains were streaked and grown on R2YE agar plate for three days. And a plug of growing cells was picked up every day and impregnated with osmium. The conducting specimens were taken to McMaster electron microscopy facility for further sample preparation (fixation and dehydration) and detected under scanning electron microscope (TESCAN, VP, SEM; SEM HV: 20.00 KV).
Appendix 1:

A1.1 Construction of luxCDABE reporter plasmid for high G-C content bacteria

The routine molecular assessments of gene expression (e.g. S1 nuclease analysis, primer extension, RT-PCR, etc.) require the isolation of specific mRNA or chromosomal DNA in an undegraded form for quantitative analysis. However, these approaches are labour-intensive or difficult in Streptomyces and other Actinobacteria. Therefore, we decided to use a transcriptional reporter system to monitor gene transcription in vivo (e.g. actAB) in different genetic background for my project. One commonly used reporter system is the egfp gene which encodes enhanced green fluorescent protein\textsuperscript{177}, and it has successfully assisted us to characterize some essential protein localization important for Streptomyces morphological development\textsuperscript{177,178}. However, it tends to photo-bleach rapidly, often compromising report weak signals from temporal controlled promoters. This is further exacerbated by the significant autofluorescence by Streptomyces itself. Other reporter genes frequently used in gram-negative and some gram-positive bacteria, such as lacZ, amy, xylE and melC, all have their own advantages and disadvantages\textsuperscript{179-181}.

Later on, luxAB genes from Vibrio harveyii, which encode a heterodimer of luciferase, were employed in Streptomyces. However, the luminescence visualization requires the addition of n-decanal substrates which may not be able to pass through the cell wall of all Streptomyces cell types in equivalent efficiency. Therefore, it came to our mind to adapt the entire gene cluster of luminescence production into Streptomyces,
which includes genes encoding luciferase (\textit{luxAB}) as well as genes (\textit{luxC}, \textit{luxD}, and \textit{luxE}) encoding enzymes for the substrates (tetradecanal) biosynthesis \cite{156,182}.

The biggest challenge to apply luminescent reporter genes which are originated from gram-negative bacteria into \textit{Streptomyces} is the difference in codon usage. \textit{Streptomyces} has high GC content (> 70\%) and favours G or C residues in the wobble position of most translational codons\cite{127}. Furthermore, in \textit{S. coelicolor}, there is only one tRNA for leucine-encoding TTA codon and it is encoded by \textit{bldA} gene which is developmentally regulated such that the abundance of its product is very low during early growth of the culture and expressed poorly in liquid culture \cite{132,183,184}. Unfortunately, the \textit{luxCDABE} operon we would use was adapted from a terrestrial bioluminescent bacterium \textit{Photorhabdus luminescens} which uses codons rich in A or T in their wobble positions and consists of 63 TTA codons in \textit{lux} genes\cite{182}. This would hamper translational efficiency and luminescence expression has to be subject to the \textit{bldA} regulation that would devalue their experimental utilities. Therefore, it is necessary to construct an entirely synthetic \textit{luxCDABE} gene cluster that replaces the TTA codon with codon that ends with G or C in the majority of its codons. This project was started by our previous exchange student, Tobias Hohenauer, and completed by PhD student Arryn Craney and I\cite{156}.

Details of the reporter construction can be referred to the publication\cite{156}. In general, to optimize expression in \textit{Streptomyces}, Tobias designed and synthesized GC enriched \textit{luxC}, \textit{luxD}, \textit{luxA}, \textit{luxB} and \textit{luxE} sequences \textit{in silico}, each of which was flanked by restriction endonuclease recognition sites for assembly into one operon and preceded by a Shine-
Dalgarno sequence. The resultant fragments of each lux gene were then introduced into Gateway entry vector pDONR221 using bacteriophage λ integrase (Invitrogen). A number of alleles were sequenced and the one that contained the least sequence alterations (e.g. insertion, deletion or point mutation) was identified for each cloned lux gene. Arryn Craney and I corrected these errors using site-directed mutagenesis (Stratagene) and I assembled individual lux genes to a luxCDABE operon in cloning vector pBluescript II SK+. The assembled operon was excised as a 5668 bp fragment and ligated into the pRT801 vector. Two transcriptional terminators (tfd and to respectively) were transferred from pIJ8660 as restriction fragments and cloned into pRT801 to flank the lux operon, creating the new vector of luminescent reporter, pMU1, which can be conjugated directly from E. coli to S. coelicolor and integrated into chromosome by site-specific recombination for single copy expression. Our lab has developed several derivatives of pMU1 to enhance the expression of luminescence and stability in the Streptomyces chromosome.

A1.2 Luminescent activities directed by promoters from actinorhodin gene cluster

To test the expression of this synthetic luminescent reporter during actinorhodin biosynthesis, I introduced DNA fragments upstream of luxCDABE containing promoter of hrdB gene which encodes a house-keeping sigma factor in S. coelicolor and 9 putative promoters from act gene cluster.

As previously indicated, the act gene cluster consists of three regions (Figure 2.1 and Figure A1.1A): the mid actII region for regulation and export, the right wing for
backbone construction and immediate modification (\textit{actIII}, \textit{actI}, \textit{actVII}, \textit{actIV} and \textit{actVB})
\cite{135}, and the left wing of \textit{actVI} and \textit{actVA} operons for late tailoring steps\textsuperscript{139, 144}. Transcripts
have been characterized for most \textit{act} genes by S1 mapping or other conventional genetic
molecular approaches. All \textit{act} genes, except for \textit{actVI-orfA}, \textit{actII-orf1} (\textit{actR}) and \textit{actIII},
are transcribed rightward: (1) \textit{actII-4}, the gene encoding pathway specific activator, is
directed by its own promoter (\textit{P}\textsubscript{actII-4})\textsuperscript{134}. (2) The inter-genetic region between \textit{actIII} and
\textit{actI-orf1} contains two divergent promoters directing leftward \textit{actIII} (\textit{P}\textsubscript{actIII}) and the
rightward operon of \textit{actI-orf1/orf2/orf3}, \textit{actVII}, \textit{actIV} and \textit{actVB} (\textit{P}\textsubscript{actI}), respectively\textsuperscript{135}. (3)
Another divergent pair of promoters is located at left end of the cluster between \textit{actVI-
orfA} (\textit{P}\textsubscript{actVI-orfA}) and \textit{actVI-orf1} (\textit{P}\textsubscript{actVI-orf1})\textsuperscript{139}. (4) A third pair of divergent promoters is
identified between \textit{actR} (\textit{P}\textsubscript{actR}) and \textit{actII-orf2/orf3} (\textit{P}\textsubscript{actAB})\textsuperscript{132}. (5) However, the promoter
distribution within \textit{actVI} and \textit{actVA} regions was not clear. One (\textit{P}\textsubscript{actVI-orf3}) may locate
within the 67 bp intergenetic region between \textit{actVI-orf2} and \textit{orf3}. The other (\textit{P}\textsubscript{actVA-orf3}) is
likely within the 95 bp intergenetic region between \textit{actVA-orf2} and \textit{orf3}. I fused these 9
putative promoters to \textit{luxCDABE} gene cluster, introduced them into wild type M145.
Luminescence was tested in ACT production medium for 5 days and a strain containing
the empty pMU1 vector was used as a control (Figure A1.1B, solid blue and red lines
respectively). I also measured the total yields of actinorhodin from M145 and a \textit{ΔactI} null
mutant (Figure A1.1B, dashed blue and red line respectively).
Figure A1. 1 act promoter activities and actinorhodin production in M145

(A) 9 putative promoters (black turning arrows) were indicated within act gene cluster. (B) Luminescence directed by $P_{act}$ is shown by solid blue lines while luminescence from
strains bearing empty vector shown by solid red lines. Actinorhodin productions were measured in M145 (dashed blue lines) and ΔactI (dashed red line).

Consistent with the previous test from our lab\textsuperscript{156}, I observed no background luminescence in strains containing promoterless lux operon. In contrast, the P\textsubscript{hrdB} dependent luminescence was clearly detected at 16 hours and well above that of the promoterless control.

Detectable luminescence was achieved from all act promoter fusions and activation of structural genes (e.g. actI operon) was not distinct from that of tailoring genes (e.g. actVI-orf1). Both sets of promoters were turned on by 16-20 hour. The luminescence expressions were comparable to or even higher than that directed by P\textsubscript{hrdB} and peaked by ~40 hour prior to the detectable yield of actinorhodin. Furthermore, the active P\textsubscript{actVI-orf3} and P\textsubscript{actVA-orf3} suggest that the last two genes (actVI-orf3 and orf4) in actVI region are cotranscribed with the first two genes (actVA-orf1 and orf2) in actVA region, agreed with the functions their products played in succession in late tailoring steps. As actVA-orf1 encodes a transmembrane protein that may serve as an accessory ACT transporter (ActC), its co-transcription with genes involved in biosynthesis of (S)-DNPA and 6-DDHK suggests possible coordination between export/resistance system and late tailoring steps of actinorhodin biosynthesis. In addition, the performance of P\textsubscript{actR} and P\textsubscript{actAB} is of great interest as I would like to investigate the mechanism of coordination between export/resistance and actinorhodin biosynthesis using the ActR-ActA/ActB model. Basal level of actR expression was detected by 16 hour and reached its maximum before actionrohdin accumulated in the medium. As actR appeared to be auto-repressed\textsuperscript{133}, the
steep rise of its promoter activity which was synchronous with the induction of biosynthetic gene expression might be a hint of relief on autorepression by pathway intermediates. In contrast, $P_{actAB}$ was not turned on until 40 hours and boosted along with the actinorhodin production. All these preliminary results successfully sketched a brief profile of $act$ gene transcriptions through different stages of actinorhodin production and I would use this synthetic $luxCDABE$ reporter to investigate the mechanism of correlation between actinorhodin biosynthesis and export/resistance in $S. coelicolor$.

Table A1. 1 Bacterial strains and plasmids used in Appendix 1

<table>
<thead>
<tr>
<th>Strain/plasmid/cosmid</th>
<th>Description/function</th>
<th>Source/Creator</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S. coelicolor$ M145</td>
<td>Wild type host strain, SCP1*, SCP2*</td>
<td>John Innes Centre</td>
</tr>
<tr>
<td><em>E. coli</em> ET12567/pUZ8002</td>
<td>Host strain for DNA conjugation, containing the non-transmissible $oriT$ mobilizing plasmid pUZ8002 $dam13::Tn9$, $dcm-6$, $hsdM$, $hsdR$, $recF143$, $zijj-201::Tn10$, $galK2$, $galT22$, $ara14$, $lacY1$, $xyl-5$, $leuB6$, $thi-1$, $tonA31$, $rpsL136$, $hisG4$, $tsx-78$, $mtlI$, $glnV44$</td>
<td>Kieser et al. (2000)</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>Host strain for DNA cloning $fhuA2Δ(argF-lacZ)U169$, $phoA$, $glnV44$, $Φ80Δ(lacZ)M15$, $gyrA96$, $recA1$, $relA1$, $endA1$, $thi-1$, $hsdR17$</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRT801</td>
<td>Integrative vector for Streptomyces; $oriT(RK2)$, int, $attP(ΦBT1)$, $aac(3)IV$</td>
<td>Gregory et al. (2003)</td>
</tr>
<tr>
<td>pMU1</td>
<td>Promoterless $luxCDABE$ operon, flanked by transcriptional terminators, preceded by in-frame stop codons and ribosome binding sites, $aac(3)IV$</td>
<td>Craney et al. (2007)</td>
</tr>
<tr>
<td>pMU1-hrdBpux</td>
<td>$hrdB$ promoter transcriptional fused to $Yeu$ $luxCDABE$</td>
<td>Ye Xu</td>
</tr>
<tr>
<td>pMU1-actII-orf4pux</td>
<td>actII-orf4 promoter transcriptional fused to $Yeu$ $luxCDABE$</td>
<td>Ye Xu</td>
</tr>
<tr>
<td>pMU1-actIpux</td>
<td>actI promoter transcriptional fused to $Yeu$ $luxCDABE$</td>
<td>Ye Xu</td>
</tr>
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</table>
**Table A1. 2 Oligonucleotide primers used to build P_{act} directed lux reporter**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrdBpF</td>
<td>GATCGC GGATCC</td>
<td>Forward primer for hrdb promoter cloning</td>
</tr>
<tr>
<td></td>
<td>GTTGGCTCGGACTGTCGTCATC</td>
<td></td>
</tr>
<tr>
<td>hrdBpR</td>
<td>GTCGGC GGTAAC</td>
<td>Reverse primer for hrdb promoter cloning</td>
</tr>
<tr>
<td></td>
<td>GCCGACCTCAACTCCCTTGA</td>
<td></td>
</tr>
<tr>
<td>actII-orf4pF</td>
<td>GCCTCG GGATCC</td>
<td>Forward primer for actII-orf4 promoter cloning</td>
</tr>
<tr>
<td></td>
<td>ACCACTGCTCTCGGTAAAAT</td>
<td></td>
</tr>
<tr>
<td>actII-orf4pR</td>
<td>GTTGAT GGTTAC</td>
<td>Reverse primer for actII-orf4 promoter cloning</td>
</tr>
<tr>
<td></td>
<td>TACATGGACACGTCCCAATAAG</td>
<td></td>
</tr>
</tbody>
</table>

1. "kan"^\textsuperscript{R}, kanamycin resistant

2. "Expression of protein for DNA conjugation in trans, kan"^\textsuperscript{R}

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequences</th>
<th>Reverse Sequences</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>actIpF</td>
<td>AACCGG GGATCC GATCTCCAGCCGATTC</td>
<td></td>
<td>Forward primer for actI promoter cloning</td>
</tr>
<tr>
<td>actIpR</td>
<td>AGCAGC GGATCC ACCCATCTCCTTCGAC</td>
<td></td>
<td>Reverse primer for actI promoter cloning</td>
</tr>
<tr>
<td>actIIIpF</td>
<td>AACCGG GGATCC GATCTCCAGCCGATTC</td>
<td></td>
<td>Forward primer for actIII promoter cloning</td>
</tr>
<tr>
<td>actIIIpR</td>
<td>AGCAGC GGATCC ACCCATCTCCTTCGAC</td>
<td></td>
<td>Reverse primer for actIII promoter cloning</td>
</tr>
<tr>
<td>actVI-orf1pF</td>
<td>CTGGTG GGATCC GCCGTAGAACGACCTC</td>
<td></td>
<td>Forward primer for actVI-orf1 promoter cloning</td>
</tr>
<tr>
<td>actVI-orf1pR</td>
<td>GCCCGG GGATCC ATCACTGTCACGGTGCTCAT</td>
<td></td>
<td>Reverse primer for actVI-orf1 promoter cloning</td>
</tr>
<tr>
<td>actVI-orfA pF</td>
<td>CTGGTG GGATCC GCCGTAGAACGACCTC</td>
<td></td>
<td>Forward primer for actVI-orfA promoter cloning</td>
</tr>
<tr>
<td>actVI-orfA R</td>
<td>GCCCGG GGATCC ATCACTGTCACGGTGCTCAT</td>
<td></td>
<td>Reverse primer for actVI-orfA promoter cloning</td>
</tr>
<tr>
<td>actVI-orf3pF</td>
<td>GCAGGG GGATCC CTGAAACGACCAGGCATTC</td>
<td></td>
<td>Forward primer for actVI-orf3 promoter cloning</td>
</tr>
<tr>
<td>actVI-orf3pR</td>
<td>GTACTG GGATCC GCCGTAGAACGACCTC</td>
<td></td>
<td>Reverse primer for actVI-orf3 promoter cloning</td>
</tr>
<tr>
<td>actVA-orf3pF</td>
<td>CGCGAC GGATCC GTCTGACCCAGCACCCTTC</td>
<td></td>
<td>Forward primer for actVA-orf3 promoter cloning</td>
</tr>
<tr>
<td>actVA-orf3pR</td>
<td>CCCGCC GGATCC GAACTCCTCATGGAAGATCTCTGT</td>
<td></td>
<td>Reverse primer for actVA-orf3 promoter cloning</td>
</tr>
<tr>
<td>actABpF</td>
<td>TCCTCG GGATCC GCTTCAACGGAACTCATGG</td>
<td></td>
<td>Forward primer for actAB promoter cloning</td>
</tr>
<tr>
<td>actABpR</td>
<td>CTTTCG GGATCC GACACGTGCTCCTCATCGTA</td>
<td></td>
<td>Reverse primer for actAB promoter cloning</td>
</tr>
<tr>
<td>actRpF</td>
<td>CATGGGGGATCC CGCTCTCGGGGAGTAAC</td>
<td></td>
<td>Forward primer for actR promoter cloning</td>
</tr>
<tr>
<td>actRpR</td>
<td>CCCACGGGGAATACCTCCTTC</td>
<td></td>
<td>Reverse primer for actR promoter cloning</td>
</tr>
</tbody>
</table>

* restriction site is underlined.
Appendix 2

ActR is one of the TetR family regulators (TFR) with solved crystal structures in both the apo form and in complex with known biologically relevant ligands\textsuperscript{185, 186}. It represses expression of \textit{actAB} encoded actinorhodin transporter by binding to its promoter region, which can be relieved by small molecules, such as actinorhodin and its relevant precursors from its biosynthetic pathway (e.g. (S)-DNPA and DHK)\textsuperscript{116}. Importantly, previous \textit{in vitro} studies showed that intermediate such as (S)-DNPA had higher affinity to ActR and was more efficient than actionrhodin in releasing ActR from the operator\textsuperscript{66, 115}. Consistent with this, P\textsubscript{actAB} was activated in cognate biosynthetic mutants of these intermediates (Chapter II, Figure 2.9), suggesting that these 3-ring molecules can derepress \textit{actAB} expression \textit{in vivo}. To test if these molecules serve as the primary intracellular signals for \textit{actAB} activation in wild type \textit{S.coelicolor}, I created an ActR mutant that responds only to the tri-cyclic intermediates (e.g. (S)-DNPA) but not final product actinorhodin.

A2.1 Structural information of apo-ActR and ligand binding ActR

In order to manipulate ligand binding specificity of ActR, the interaction between ligands (3-ring intermediates or 6-ring actionrhodin) and the protein was analyzed on our crystal structures of (S)-DNPA and actinorhodin bound ActRs respectively\textsuperscript{116}. As a typical member of TFR, ActR is a Ω-shaped homodimer constituted of 11 α helices in each monomer (Figure A2.1). Helices from α1 through the N-terminus of α4 compose the DNA binding domain (DBD). It was suggested that a pendulum-like motion of DBD led
by pivoting around the C-terminus of helix α4 is critical for its action\textsuperscript{116}. The rest of α4 through helix α8 and helix α11 make up the core of the ligand binding domain (LBD)\textsuperscript{116}. Notably, the loop between helices α6 and α7 appeared to be the most structural variable region within the core LBD and might play a critical role in ActR derepression\textsuperscript{116}. The kinked helix α9 and helix α10 form an arm that extends from one LBD and wraps around the counterpart of the opposing monomer\textsuperscript{116}.

**Figure A2.1 Structure of apo-ActR**

ActR is a homodimer protein and consists of 11 α helices in each monomer. N-terminus of this protein (from α1 to the N-terminal α4) is the DNA binding domain (DBD). C-terminus of ActR forms ligand binding domain (LBD) (C-terminal α4 to α8 plus α11) and the arm wrapping around the opposing monomer (α9 and α10). All α helices are indicated in one monomer and DBD and LBD are coded by different colours.

A tunnel runs horizontally from the outside edge of each LBD through the core to the dimer interface with the central termini slightly offsets from that of the other tunnel, and creates a cavity of about 800 Å\textsuperscript{3} within the respective monomer (Figure A2.2)\textsuperscript{116}. Two ligand binding sites were identified in each tunnel, one “proximal” and one “distal” to the dimer interface where the triggering molecules may make access to each tunnel. Each site
binds either one (S)-DNPA-like 3-ring molecule or tricyclic half of the actinorhodin molecule. A close inspection on the residues surrounding the ligand binding tunnel reveals a longitude distribution that forms a bilateral symmetric pattern of polar, hydrophobic, polar environment (Figure A2.2): the mid-point of the tunnel between proximal and distal sites is enriched by a ring of 17 hydrophobic residues, which locks the central four non-hydrogen carbons (C9, C10 and C9’, C10’) of actinorhodin into a hydrophobic core position.

Figure A2.2 Orientation of ActR ligand binding pocket
Carbons in pocket residues are coded green while oxygen, nitrogen and sulphur involved in hydrogen bonding are coded red, blue and yellow respectively. The backbone of actinorhodin and (S)-DNPA were in blue and orange respectively. Distribution of residues in ligand binding pocket follows a symetric pattern with polar proximal and distal sites and a hydrophobic mid-point, which correlates well with the structure of the two types of ligand molecules. The C-C connector of actinorhodin is locked in the mid point and its two halves fit into proximal and distal sites where they are stabilized by the network of hydrogen bonds. Two (S)-DNPA molecules sit in the pocket in a back to back orientation. Therefore, each ligand binding pocket accommodates either one actinorohdin or two (S)-DNPAs.
By contrast, the proximal (Figure A2.3A) and distal sites (Figure A2.3B) are surrounded by residues with higher hydrophilic side-chains or backbone atoms. In addition, the very end of the proximal site (Figure A2.3C) is constituted by charged residues from the opposite monomers (E170’, E173’, R177’ and R225’). Such an environment provides an extensive hydrogen-bonding network to stabilize actinorhodin’s two polar oxygen-containing molecular halves, or to situate two 3-ring intermediates ((S)-DNPA) in a back-to-back fashion. Therefore, each ligand binding domain of ActR dimer binds one molecule of actinorhodin, or two molecules of the intermediates with one proximal and one distal to the dimerization interface.

Figure A2.3 Constitution of binding sites in ligand pocket of ActR
(A) A sectional view from the proximal access (at the interface of the two monomers) of the ligand binding pocket. (B) A sectional view from the distal site of the ligand binding pocket (outside edge of the tunnel). (C) Residues (R225’, R177’, E173’ and E170’) at the very end of the proximal site are extruded from the opposing monomer and also contribute to the hyrophilic environment of the proximal site.
Previous studies showed that the ActR ligand-binding cavity can accommodate diverse synthetic 3-ring anthraquinone molecules that confer similar inducing ability to the natural actinorhodin precursors\textsuperscript{115}. In fact, when superimposing the structures bound with actinorhodin or (S)-DNPA, the two tri-cyclic halves of actinorhodin do not occupy exactly the same positions as (S)-DNPA. Rather, the 3-ring precursors can adapt to more flexible ActR conformations that are not accessible to actinorhodin due to its rigid carbon-carbon bond that connects the two halves\textsuperscript{115, 116}. This might explain why the tri-cyclic pathway intermediates and some synthetic 3-ring molecules have higher affinity to ActR and can interrupt ActR-DNA interaction at lower concentrations. To create an “intermediate responsive only” ActR mutant, we decide to introduce residues with larger side chains into the pocket, hence create steric hindrance at the binding sites occupied by the proximal or distal half of actinorhodin or by its central covalent bond, and prevent a productive interaction. I reasoned that (S)-DNPA might still be able to interact with some of these ActR mutants as it is unconstrained by the central C-C bond.

**A2.2 Point mutations made in ActR ligand binding pocket**

As shown in Table A2.1 and Figure A2.4A, 10 residues within the LBD core that are important in stabilizing actinorhodin but might be dispensable for accommodating tri-cyclic intermediates were selected and 20 point mutations were made on them. For residues that reside in the distal binding site, V90 and V92 are located within the loop between helices α4 and α5, M107 and L111 are on helix α5, G135, G138 and T142 are on helix α7. Except for T142 which has a hydroxyl-side chain, all these residues are
relatively small non-polar neutral amino acids that contribute their amine or carboxylic acid groups to hydrogen bondings which could be interrupted by replacing with bulkier residues (e.g. with benzyl- side chain) or amino acids with different polarity. Remarkably, G138 and T142 were considered to be most critical as they are closer to the ligand and provide sturdy hydrogen bonds that enhance the ligand orientation in the structural model. Therefore, more varieties and different combination of mutations were made on them (e.g. G138L/A/W/Y/F, T142Y/W, GTWY, GTYW, GTWW). For the proximal site, I replaced two residues, G160 and S164, with bulkier ones (tyrosine or tryptophan) to introduce stereo-hindrance. Furthermore, as our structural model suggested, R225’ extruded from the opposing monomer was anchored in the vicinity of the ligand’s carboxylic group and a modest conformational shift might push its charged side chain close enough to the edge of the proximal site so as to interact with the molecule. Therefore, I changed this residue to the small non-polar alanine to abolish its potential ability to form hydrogen bond with the ligand. Taken together, I expected that the access to either binding site is hindered by these mutations so that the binding or the entry of the “inflexible” actinorhodin would be able to impeded, while 3-ring intermediates would adjust their orientations to avoid “crash” with the bulkier residues and maintain their normal interaction with ActR (Figure A2.4B).

**TableA2. 1 List of ActR mutants**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>helix</th>
<th>Ligand binding pocket</th>
<th>Protein purification</th>
<th>DNA Binding</th>
<th>ACT</th>
<th>(S)-DNPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>V90W</td>
<td>α4/α5</td>
<td>Distal</td>
<td>Yes</td>
<td>-</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>V92W</td>
<td>$\alpha4/\alpha5$</td>
<td>Distal</td>
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<td>V92Y</td>
<td>$\alpha4/\alpha5$</td>
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<td>induction</td>
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<td>M107W</td>
<td>$\alpha5$</td>
<td>Distal</td>
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<td>N/A</td>
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<td>$\alpha5$</td>
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<td>G135A</td>
<td>$\alpha7$</td>
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<td>G138L</td>
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<td>G160W</td>
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<td>N/A</td>
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<td>-</td>
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<td>N/A</td>
</tr>
<tr>
<td>S164Y</td>
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<td>-</td>
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<td>N/A</td>
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<td>insoluble</td>
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<td>N/A</td>
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</table>

*DNA binding ability was compared between wild type ActR and mutants with increased protein concentrations (2nM, 5nM, 10nM, 20nM and 50nM). As the wild type ActR can tightly bind DNA at concentration as low as 5nM, any mutant that showed evident disparity in DNA binding at this concentration was eliminated. Therefore, mutants that bound DNA at concentration below 10nM were considered as “comparable to wild type” and were marked as positive (+) candidates for the following ligand specificity test.
FigureA2. 4 Mutations made in ligand binding pocket of ActR
(A) Ten residues closely surrounding the ligand were selected for mutations. (B) A mutation at T142 (T142W) would introduce a bulky side chain that conflicts with actinorhodin (blue), but the interruption might be less severe with (S)-DNPA (orange) as it is situated in an orientation slightly deviated from the “crash” and might adopt more flexibility to avoid the interruption.

Among 16 alleles that generated protein of the correct size and similar yields in comparison to the wild type ActR, seven of them (V90W, V92W, M107W, GTWY, G160W, S164W and S164Y) showed significant decrease in their ability to bind promoter sequence as tested by gel mobility shift assays(Table A2.1). The remaining nine mutant proteins (V92Y, L111W, G135A, G138W, G138L, T142Y, T142W, GTYW and GTWW) bound DNA with affinity similar to that of the wild type protein (Table 2.1, Figure A2.5).
However, only T142W and GTYW failed to respond to actinorhodin but could still release DNA in response to (S)-DNPA (Figure A2.6).

Figure A2.5 DNA binding abilities of nine ActR mutants
Nine site-directed mutants of ActR have the capacity to bind and shift the electrophoretic mobility of an actO probe. These are proteins that could be purified and that retained the ability to interact stably with DNA. Lanes labeled P contain probe only; ActR indicates wild type proteins and the mutations listed in the remaining lanes indicate the specific site-directed mutants. All proteins were loaded at 5nM.

Figure A2.6 ActR mutants that are responsive to the (S)-DNPA but not actinorhodin
Wild type ActR was incubated with operator DNA in the absence of ligand (lane C) and in the presence of 250 µM actinorhodin, aloesaponarin II and (S)-DNPA (lanes 1-3 respectively). The two mutant proteins were incubated with DNA in the absence of ligand
(C2), in the presence of DMSO to control for solvent effects (C3) and in the presence of actinorhodin or (S)-DNPA at the indicated concentrations. DNA binding by ActR is eliminated by 250 \( \mu \text{M} \) actinorhodin, (S)-DNPA and partially relieved by the shunt product aloesaponarin II, which is not a good ligand at lower concentrations\(^6\). However, DNA binding by the T142W mutant and the GTYW mutant (which includes the T142W sequence change) was prevented by (S)-DNPA but not actinorhodin, suggesting that they had retained a productive interaction with the intermediate but not with the mature antibiotic. (S)-DNPA reduces DNA binding by both the T142W and GTYW mutants at concentrations as low as 10 \( \mu \text{M} \) and eliminates it at 50 \( \mu \text{M} \). In contrast, actinorhodin has no effect on DNA binding by these mutants even at concentrations as high as 200 \( \mu \text{M} \).

**A2.3 in vivo ligand response by T142W and GTYW mutants**

The wild type \( \text{actR} \) or the mutant allele (T142W or GTYW) along with \( \text{actR-actA} \) intergenetic region was fused with \( \text{luxCDABE} \) and introduced into \( \text{S.coelicolor} \) with \( \text{actR} \) and \( \text{actI} \) deleted from \( \text{act} \) gene cluster (see method and material in Chapter II). These reporter strains were fed with actinorhodin or (S)-DNPA at concentration from 10 nM to 1\( \mu \text{M} \) (Figure A2.7). Consistent with *in vitro* assay, the \( \text{actR} \) mutants could respond only to the 3-ring molecule (S)-DNPA and would be further tested during ACT biosynthesis (Chapter II, Figure 2.9).
Figure A2.7 *in vivo* response to added ActR ligands

Timecourse response of reporter strains expressing either ActR (purple) or the mutant alleles (T142W-cyan, GTYW-orange) to (A) actinorhodin or (B) (S)-DNPA at varied concentrations (from 10nM to 1µM). The ligand dissolvent DMSO was also added to the
reporter strains as a control. In the presence of actinorhodin (A), only reporter controlled
by WT ActR was induced, while both types of reporter strains could be activated by (S)-
DNPA (B).

A2.4 Method and Material

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<th>Description/function</th>
<th>Source/Creator</th>
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<td></td>
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<td>pTOPO-actR</td>
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<td>Tahlan et al. (2007)</td>
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\( pO^{\text{act}} \) For amplification of ActR binding operator sequence (\( \text{kan}^R \)) Tahlan et al. (2007)

**Cosmids**

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<tr>
<td>BAC28G1</td>
<td>Bacterial artificial chromosome containing ( \text{act} ) gene cluster</td>
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\( \text{amp}^R, \) ampicillin resistant; \( \text{apr}^R, \) apramycin resistant; \( \text{cam}^R, \) chloramphenicol resistant; \( \text{kan}^R, \) kanamycin resistant; \( \text{spc}^R, \) spectinomycin resistant

4. **Table A2.** 3 Oligonucleotide primers used in building and testing ActR mutations

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<th>Name</th>
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<tr>
<td>AR-TP1F</td>
<td>CACCGTGTCGCGAAGCGAGGAAGGGAGGC</td>
<td>Forward primer for cloning point mutated actRs in pET151</td>
</tr>
<tr>
<td>AR-TP1R</td>
<td>TCA TGACTCCGCGGGGGCGATCC</td>
<td>Reverse primer for cloning point mutated actRs in pET151</td>
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<td>AR-V90WR( ^a )</td>
<td>CGGGACCTCCCACCTCGGTAGCTCG</td>
<td>Reverse internal mutagenic primer for ( \text{actR}^{V90W} ) by megaprimer mutagenesis</td>
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<tr>
<td>AR-V92WR</td>
<td>CCGGTTCGCGCCTCCACCTCGGTTC</td>
<td>Reverse internal mutagenic primer for ( \text{actR}^{V92W} ) by megaprimer mutagenesis</td>
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<tr>
<td>AR-V92YR</td>
<td>CCGGTTCGCGGTTACTCCATCTCGGTTC</td>
<td>Reverse internal mutagenic primer for ( \text{actR}^{V92Y} ) by megaprimer mutagenesis</td>
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<tr>
<td>AR-M107WR</td>
<td>CCGCCGGACCCACTCCGACCTGACCTG</td>
<td>Reverse internal mutagenic primer for ( \text{actR}^{M107W} ) by megaprimer mutagenesis</td>
</tr>
<tr>
<td>AR-L111WR</td>
<td>CATCCTCCGACCGACCGGCCACATC</td>
<td>Reverse internal mutagenic primer for ( \text{actR}^{L111W} ) by megaprimer mutagenesis</td>
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<tr>
<td>AR-G135AF</td>
<td>GGACCCCAAACGCGATGGTGGGGATGG</td>
<td>Forward internal mutagenic primer for ( \text{actR}^{G135A} ) by megaprimer mutagenesis</td>
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<tr>
<td>AR-G138AF</td>
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<td>AR-G138FF</td>
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A2.4.1 Mutagenesis of ActR

Site directed megaprimer mutagenesis (Tyagi et al. 2004) was employed to introduce point mutations into actR gene. Internal mutagenic primers and corresponding forward

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<td>AR-T142WF</td>
<td>GGATGGGAGCGCTGGATGAACCTGCTGC</td>
<td>Forward internal mutagenic primer for actR&lt;sub&gt;T142W&lt;/sub&gt; by megaprimer mutagenesis</td>
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<tr>
<td>AR-T142YF</td>
<td>GGATGGGAGCGCTACATGAACCTGCTGC</td>
<td>Forward internal mutagenic primer for actR&lt;sub&gt;T142Y&lt;/sub&gt; by megaprimer mutagenesis</td>
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<td>AR-GTYWF</td>
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<td>AR-GTWWF</td>
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<td>Forward internal mutagenic primer for actR&lt;sub&gt;GTWW&lt;/sub&gt; by megaprimer mutagenesis</td>
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<td>AR-S164WF</td>
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<td>Forward internal mutagenic primer for actR&lt;sub&gt;S164W&lt;/sub&gt; by megaprimer mutagenesis</td>
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<td>AR-R225AF</td>
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<tr>
<td>TET-EMSA-R</td>
<td>TCATATTTGCCATCCATTTCG</td>
<td>Reverse primer for EMSA probe</td>
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</table>

<sup>*</sup> Point mutation made in actR is boldfaced.
and reverse primers are listed in Table A2.3 (mutations are boldfaced). 50 μl reactions were employed containing 50 pmoles each primer, 50 ng template 50 μM dNTPs, 1x Vent Buffer, 10mM MgSO₄, 5% DMSO and 2.5 units of Vent DNA polymerase.

The generated PCR product (megaprimer) was purified and eluted in 10 μl. All 10 μl megaprimer was used to pair with the other flanking primer in the second round of PCR, giving rise to the final product with pointed mutation. 50 μl reactions were employed containing 50 pmoles of the other flanking primer, 10 μl megaprimer, 50 ng Template, 50 μM dNTPs, 1x Vent Buffer, 10mM MgSO₄, 5% DMSO and 2.5 Units Vent DNA polymerase.

As the forward flanking primer AR-TP1F (see Table A2.3) contains four bases (CACC) at 5’ end, the final PCR product was cloned directly into pET151 by directional TOPO cloning (Invitrogen) and transformed into \textit{E. coli} TOP10 cells (see Table A2.2). Ampicillin resistant transformants were selected. DNA were isolated and sequenced by T7 forward/terminal primers (Mobix lab, McMaster University).

\textbf{A2.4.2 Protein purification}

Wild type and mutant ActR proteins (his6-tagged) were expressed in IPTG-induced (1 mM) 1 L cultures of \textit{E. coli} BL21 Star™ (DE3) grown at 37 °C. Cells were pelleted by centrifugation, resuspended in BugBuster protein extraction reagent (5 ml per gram wet cell paste) and Benzonase (25 units/ml) (Novagen). Cells were lysed with shaking at room temperature for 20 min, cell debris was removed by centrifugation at 1200×rpm for 30 min at 4 °C. Supernatants were filtered through 0.45μm disc (PALL life Science) and incubated with 3 ml Ni-NTA Agarose Resin (Qiagen) for 1 hour at 4 °C. The mixture was
loaded onto a column, washed with 20ml of wash buffer (10 mM Tris-HCl, 150 mM NaCl and 50 mM imidazole, pH 7.9) three times and then eluted with elution buffer (10 mM Tris-HCl, 150 mM NaCl and 0.5M imidazole, pH 7.9). His\textsuperscript{x6}-tagged protein was transferred to storage buffer (10 mM Tris-HCl, 500 mM NaCl, 20% glycerol, pH 7.9) using PD-10 Desalting Column (GE Healthcare). Protein concentration was determined by Bradford assay (Bio-Rad).

### A2.4.3 Electrophoresis Mobility Shift Assay

EMSA was carried out as described before (O’Connor et al, 2002), but EDTA was omitted from the binding buffer and 10% polyacrylamide gels containing 1.5% were used for analysis. The primers TET-EMSA-F and TET-EMSA-R were used to amplify ActR operator sequence from pO\textsuperscript{act} (Tahlan et al. 2007). This DNA fragment was end labeled using [γ\textsuperscript{32}P] (Sambrook et al. 1989). Actinorhodin and (S)-DNPA were dissolved in DMSO.
Appendix 3

A3.1 Undecylprodigiosin biosynthesis in *S. coelicolor*

*S. coelicolor* produces the red pigmented prodiginine antibiotic undecylprodigiosin (red) and its cyclic derivative, butyl-meta-cyclo-heptylprodiginine \(^{187,188}\). The prodiginine gene cluster consists of 23 genes that are organized into four transcriptional units (Figure A3.1A)\(^{188}\). Most of the gene products or their homologues were annotated to function in biosynthesis of fatty acid, type I modular polyketide, non-ribosomal peptide or \(\alpha\)-oxoamine\(^{188}\). The undecylprodigiosin is proposed to be synthesized from a bifurcated pathway involving the enzymatic condensation of 4-methoxy-2,2’-bipyrrrole-5-carbaldehyde (MBC) with a 2-undecyl-pyrrole (UP)\(^{188}\). A following oxidative cyclisation converts this tri-pyrrole into its cyclic derivative.

At least 9 genes (*redM, redW, redU, redO, redX, redN, redV, redI and redF*) are proposed to involve in MBC biosynthesis\(^{189}\) (Figure A3.1 B (1)). RedM, a homologue of adenylation domain of modular non-ribosomal peptide synthase (NRPS), activates carboxyl group of L-Proline with an ATP and the so formed L-Prolyl group is transferred to the phosphopantetheine thiol of Peptidyl Carrier Protein (PCP), RedO\(^{189}\) (a). This L-prolyl-S-RedO is then converted to L-pyrrolyl-S-RedO by the FAD-dependent oxidase RedW\(^{189}\) (b). The pyrrolyl-2-carboxyl unit is then transferred from RedO to the active cysteine residue of the KS\(^C\) domain in RedX (c) and a malonyl-CoA is concomitantly transferred to one of the two Acyl Carrier Protein (ACP) domains of RedN\(^{189}\). A decarboxylative condensation of the malonyl unit to pyrrolyl-2-carboxyl-RedX follows
and this generates the β-keto-β-pyrrolyl-propanoyl thioeaster attached to RedN (d).

Next, this RedN bound intermediate is condensed with L-Serine by the pyridoxal phosphate (PLP) dependent α-oxoamine synthase (OAS) domain of RedN and yields seryl imine, which undergoes a series of reactions including cyclisation and dehydration to form 4-hydroxy-2,2′-bipyrrole-4-methanol (HBM) (e). Oxidation of the hydroxyl group of HBM is likely to be catalyzed by RedV and gives the 4-hydroxy-2,2′-bipyrrole-4-carbaldehyde (HBC) (f). Methylation of 4-hydroxyl group of this intermediate is the last step to generate MBC, which could be catalyzed by RedF or an S-adenosylmethionine dependent methyltransferase RedI (g).

UP is synthesized from a decarboxylative condensation between glycine and an acetate derivative, 3-ketomyristic acid and at least six genes (redP, redR, redQ, redL, redJ, and redK) are involved (FigureA3.1 B(2)). First, RedP, a homologue of FabH (3-ketoacyl-synthase III), loads an acetyl-CoA starter unit to a malonyl-RedQ (ACP), which is reduced by enzymes from type II fatty acid biosynthetic pathway to give butyryl-RedQ (h). Four more elongation steps are catalyzed by RedR (h) and the resultant dodecyl group is transferred to the first ACP domain of RedL, assisted by the thioesterase RedJ (i). A subsequent ketosynthase reaction with a malonyl-CoA bound to the second ACP domain of RedL generates a β-ketomyristoyl thioester (i). The PLP domain of RedL then condenses this bound intermediate with a glycine and releases it as a cyclized 4-keto-2-undecyl-4, 5-dihydropyrrole (j). RedK would catalyze the final reduction and dehydration to produce UP (k).
Finally, RedH catalyzes the condensation of MBC and UP and yields the tripyrrole product, undecylprodigiosin \(^{188}\) (Figure A3.1B (3)) (l). This could be followed by an oxidative cyclization by RedG to give butyl-meta-cyclo-heptylprodiginine \(^{188}\) (m).

Figure A3.1 Undecylprodigiosin biosynthetic pathway
(A) The “red” gene cluster. Regulatory genes are gridded. Genes involved in making MBC are light gray. Genes for making 2-undecyl-pyrrole are dark gray. Genes of
unknown function are blank. \textit{redH} for final condensing is dotted and \textit{redG} for oxidative cyclisation is wavy. Genes of unknown function are blank. (B) The bifurcated biosynthetic pathway: (1) MBC formation; (2) UP formation; (3) Condensation and cyclisation.

A3.2 SCO4008-SCO4007, a TetR-TetA like combination that may be relevant to undecylprodigiosin export/resistance in \textit{S. coelicolor}

So far, no resistance or export gene has been identified and characterized from the “\textit{red}” gene cluster. As the physiological roles of prodiginines are very complicated and still debated (e.g. acidification of cytoplasm by promotion of H+/Cl- symport, DNA intercalation and Cu$^{2+}$ binding to facilitate oxidative DNA cleavage, Cell cycle arrest, apoptosis \textit{etc.})$^{187, 192}$, it is hard to predict resistance mechanism employed by their producers. Furthermore, their precise cellular location is not clear as it was detected from both intracellular and extracellular fraction$^{188, 193}$. Most common idea is that prodigiosin is a highly hydrophobic molecule and is associated with the cell envelope (e.g. \textit{Streptomyces mycelium}), but how it is secreted is not known$^{193}$.

Coincidentally, a pair of genes, \textit{SCO4008-SCO4007}, outside the “\textit{red}” gene cluster was found relevant to the production of undecylprodigiosin in our lab. More intriguing is the fact that \textit{SCO4008} encodes a TFR and \textit{SCO4007} encodes a putative MFS transporter, which resemble the \textit{tetR-tetA} model of tetracycline resistance and the \textit{actR-actAB} model of actinorhodin resistance. A gel mobility shift experiment conducted by Dr. Kapil Tahlan showed that while the full-length SCO4008 (4008-FL) tightly bound \textit{SCO4008-4007} intergenic DNA (137 nt) at concentration as low as 5 nM, a C-terminal truncated SCO4008 (4008-CT) only barely bound the DNA at a much higher concentration (100
nM). Typically, the C-terminal part of TFR contributes to regulatory functions involved in the binding with inducing molecules. Deletion of this part could severely affect the integrity of the protein structure, hence the reduction of its DNA binding ability. In addition, Dr. Tahlan found that strains either overexpressing 4008-CT or having SCO4008 gene knocked out accumulated the red pigment in the membrane, but the biological relevance to red production by SCO4007 gene product was still speculative. All these preliminary results raised our interest in understanding the roles SCO4008 (TFR) and SCO4007 (exporter) play in red biosynthesis and export/resistance. Is SCO4007 involved in the secretion of undecylprodigiosin in S. coelicolor? Does SCO4008 negatively control the expression of SCO4007 and/or the biosynthesis of the red antibiotic? If answers to the above questions are positive and SCO4008-SCO4007 is another analogue system of TetA-TetR, how do they coordinate with biosynthetic genes in the “red” cluster? Does SCO4008 interact with the undecylprodigiosin or some of its intermediate? If not, what signaling molecules does SCO4008 respond to? To solve all these unknowns, I set out to confirm the impact of SCO4008 and SCO4007 on the red production.

A3.3 Construction of SCO4007 and SCO4008 mutants and examine their effects on “red” production

Dr. Tahlan built SCO4007 and SCO4008 deletion mutants by replacing the respective gene with an apramycin resistance marker (aac (3)IV). However, compared with the wild type strain M145, these two mutants did not show observable phenotypes such as
significant increase or decrease of the red colour associated with mutant colonies on solid medium. As the visualization of red pigment is often disturbed by actinorhodin, a blue antibiotic produced later on, to quantitatively evaluate the impact of SCO4008 and SCO4007 on the “red” biosynthesis I grew M145, ΔredD, SCO4007 and SCO4008 deletion mutants in liquid culture and extracted the red pigment from the harvested mycelium of each strain daily for four days. The undecylprodigiosin production is determined by reading absorbance at 530 nm (Figure A3.2). The ΔredD null mutant lacks the pathway specific activator of undecylprodigiosin biosynthesis and did not produce any red pigmented antibiotics as expected. Consistent with Dr. Tahlan’s observation, deletion of SCO4007, the gene that encodes a transmembrane protein, did not cause significant decrease of red production in SCO4007 deletion mutant, suggesting that this putative exporter, different from our expectation, is not actively involved (or not the major component) in the red production. However, SCO4008 mutant produced 30-40% more of undecylprodigiosin than that made by M145, suggesting that SCO4008 negatively regulates the red production in an unknown means. These results were reproducible in the new set of SCO4007 and SCO4008 mutants which I created by replacing the gene of interest with a spectinomycin resistance marker (aadA) for further characterization using the luminescent reporter system.
Figure A3.2 Undecylprodigiosin production in M145, ΔredD, SCO4007 and SCO4008 deletion mutants.

ΔredD (red) has pathway specific activator deleted from the red cluster and produces no detectable red pigments. Compared with M145 (blue), the SCO4007 deletion mutant (purple) does not show significant reduction in red production while SCO4008 mutant (green) yielded 30%-40% more undecylprodiginine.

A3.3 Activity of redP promoter is enhanced in a SCO4008 deletion mutant

To detect if the enhanced red production in the SCO4008 deletion mutant is indeed resulted from the enhanced expression of red genes, I built lux fusions with P_redD or P_redP and introduced them into a new set of SCO4008 deletion mutant in which the SCO4008 was replaced by spectinomycin resistance gene addA so that the exconjugants bearing the luminescent reporter could be selected by apramycin. The lux reporters were also introduced into M145 and the ΔredD mutant. As shown in Figure A3.1A, the transcript directed by P_redD consists of four genes, redD of pathway specific activator which is essential for structural gene expression (e.g. redE, redF and redX)\textsuperscript{24}, and three structural genes (redX, redW and redY) involved in MBC pathway; while P_redP directs a long operon composed of genes involved in both MBC and UP pathways and the final oxidative cyclization on undecylprodigiosin. As shown in Figure A3.3, P_redD was turned on in
SCO4008 mutant with comparable activity to that in M145 and was surprisingly even more active in its own deletion mutant ΔredD. In contrast, P_redP was off in ΔredD, correlating with the elimination of red biosynthesis in this mutant. However, activity of P_redP was 2-3 times higher and sustained one day longer in SCO4008 mutant than that in M145, consistent with the higher red production in the former strain and also suggested that expression of biosynthetic genes in this operon is under negative control of SCO4008 directly or indirectly. The luminescent assays were performed on a solid medium but reproducible when growing in the liquid culture.

Figure A3.3 Activation of biosynthetic genes of undecylprodigiosin in M145, ΔredD and SCO4008 deletion mutant

P_redD and P_redP were fused to lux reporter and introduced into the above specified strains. No significant disparity of P_redD activity was detected between M145 (blue) and SCO4008 deletion mutant (green), suggesting that the enhanced red production in SCO4008 mutant was not caused by a more active pathway specific activator. The P_redD was much more active in ΔredD mutant (red) which is not understood yet. Activation of P_redP was more intense and sustained longer in SCO4008 mutant than that in M145, suggesting some biosynthetic genes are under negative control of SCO4008. In the absence of this TFR, expression of these structural genes could be enhanced and started earlier, leading to increased production of the red antibiotic.

A3.4 Conclusion

Resistance and export of the red antibiotic undecylprodigiosin in its producer S. coelicolor is not yet understood and no gene from the “red” gene cluster was assigned for
such function. From previous work in our lab, a pair of protein SCO4008-SCO4007 that resembles the TFR-Export combination was found encoded outside the red gene cluster and preliminary results implicated their relevance to the red production and brought the question if they serve as the resistance system in the same manner as our ActR-ActA/ActB model. However, red yield in a deletion mutant of SCO4007 was not significantly affected, turning the role of red export by its product into a question. Interestingly, a SCO4008 deletion mutant increased red production by 30-40% and exhibited more sustained and higher activity of P_redP than that in wild type. As P_redP directs biosynthetic genes involved in the whole undecylprodigiosin biosynthetic process, their enhanced expression in the absence of SCO4008 suggested a negative regulation on red biosynthetic genes by this TFR. The mechanism of how SCO4008 influences the activity of the red gene cluster requires further investigation.

A3.5 Method and Material

Table A3.1 Bacterial strains and plasmids used in Appendix 3

<table>
<thead>
<tr>
<th>Strain/plasmid/cosmid</th>
<th>Description/function</th>
<th>Source/Creator</th>
</tr>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. coelicolor</em> M145</td>
<td>Wild type host strain, SCP1, SCP2</td>
<td>John Innes Centre</td>
</tr>
<tr>
<td><em>S. coelicolor</em> SCO4007::<em>apra</em>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>M145, SCO4007 replaced by <em>apra</em>&lt;sup&gt;2&lt;/sup&gt; cassette</td>
<td>Kapil Tahlan</td>
</tr>
<tr>
<td><em>S. coelicolor</em> SCO4008::<em>apra</em>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>M145, SCO4008 replaced by <em>apra</em>&lt;sup&gt;2&lt;/sup&gt; cassette</td>
<td>Kapil Tahlan</td>
</tr>
<tr>
<td><em>S. coelicolor</em> SCO4008::<em>spc</em>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>M145, SCO4008 replaced by <em>spc</em>&lt;sup&gt;2&lt;/sup&gt; cassette</td>
<td>Ye Xu</td>
</tr>
<tr>
<td><em>S. coelicolor</em> ΔredD</td>
<td>ΔredD deletion mutant</td>
<td>John Innes Centre</td>
</tr>
<tr>
<td><strong>E. coli</strong> ET12567/pUZ8002</td>
<td>Host strain for DNA conjugation, containing the non-transmissible oriT mobilizing plasmid pUZ8002, dam13::Tn9, dcm-6, hsdM, hsdR, recF143, zii-201::Tn10, galK2, galT22, ara14, lacY1,</td>
<td>Kieser et al. (2000)</td>
</tr>
</tbody>
</table>
E. coli BW25113  Host strain for Redirect mutagenesis  John Innes Centre

E. coli DH5α  Host strain for DNA cloning  Stratagene

\textit{E. coli} BW25113

\textit{E. coli} DH5α

\textbf{Plasmids}

\textbf{pMU1-flux}  pMU1 background with inverted \textit{luxCDABE} operon preceded by in-frame stop codons, ribosome binding sites and transcriptional terminator. The downstream transcriptional terminator was replaced by hairpin loops.

\textbf{pMU1-redDlux}  pMU1-flux, \textit{redD} promoter transcriptional fused to \textit{luxCDABE}

\textbf{pMU1-redPlux}  pMU1, \textit{redP} promoter transcriptional fused to \textit{luxCDABE}

\textbf{pIJ790}  Temperature sensitive \textit{\lambda} RED recombination plasmid  Datsenko and Wanner (2000)

\textbf{pIJ773}  Template plasmid for apramycin resistance disruption cassette in Redirect mutagenesis  John Innes Centre

\textbf{pIJ778}  Template plasmid for \textit{spc}R disruption cassette in Redirect mutagenesis (\textit{spc}R, \textit{amp}R)

\textbf{pUZ8002}  Expression of protein for DNA conjugation in trans, kan  Kieser et al. (2000)

\textbf{Cosmids}

\textbf{St10A7}  Bacterial cosmid containing \textit{SCO4007} and \textit{SCO4008} genes  John Innes Centre

\begin{tabular}{|l|l|l|}
  \hline
  \textbf{Name} & \textbf{Sequence (5'→3')} & \textbf{Function} \\
  \hline
  KOSCO4008F & AGTTGGTTACCTCAGCCGCCGGGCCT & Forward primer for deletion by \textit{SCO4008} Redirection mutagenesis \\
  & ACCCTGCGCCCATGATTCCGGGATCCGTCGACC & \\
  KOSCO4008R & CGTCCGGCGCGACCCGCGGCCGACG & Reverse primer for deletion by \textit{SCO4008} Redirection mutagenesis \\
  & GGGTCCCTCCGTGTCA & \\
  & TGAGGCTGAGCTGCTTC & \\
  KOSCO4007F & AGTTGGTTAGTGCGCGCTCGAA & Forward primer for \textit{SCO4007} deletion by \\
  & GTCGCCCGCCCATG & \\
  \hline
\end{tabular}

1. \textit{amp}R, ampicillin resistant; \textit{apr}R, apramycin resistant; \textit{cam}R, chloramphenicol resistant; \textit{kan}R, kanamycin resistant; \textit{spc}R, spectinomycin resistant

TableA3. 2 Oligonucleotide primers used in Appendix3
A3.5.1 Bacterial strains, plasmid construction and luminescent measurement conditions

Strains and plasmids used in this study are described in Table A3.1. The growth conditions and routine molecular genetic procedures for *Streptomyces* and *E. coli* were described in Experimental procedures in Chapter II.

Oligonucleotide primers listed in Table A3.2 were used to amplify red promoters that consist of the whole inter-genetic region by PCR from St10A7 cosmid. The fragment was ligated into XbaI and KpnI sites of pMU1-flux, giving rise to luminescent reporter directed by red promoter (e.g. pMU1-redDlux). This integrating plasmid was introduced into wild type *S. coelicolor* strain M145, ΔredD mutant and SCO4008 deletion mutant by conjugation. Exconjugants were selected by apramycin (50 µg/ml) and nalidixic acid (25 µg/ml), and restreaked on R2YE agar/apramycin (50 µg/ml) for making spore stocks. For luminescent tests, equal amount of *S. coelicolor* (5x 10⁵ spores) were inoculated on
96 well plates of RG5-M (agar medium and read every day for 4-5 days by VICTOR™ X Multilabel Plate Readers (Perkin Elmer).

A3.5.2 Determination of undecylprodigiosin production in *S. coelicolor*

M145, ΔredD, SCO4007 deletion mutant and SCO4008 deletion mutant were inoculated into RG5-M liquid medium at 0.5 x 10^6 spores/ml and grown for 5 days. An equal volume of absolute methanol was added to cell pellet harvested from 1ml of culture every day and mixed gently for 24 hours. After removing cell debris by centrifugation (1000 x g, 5min), the supernatant was acidified with HCl (final concentration = 0.5 M) and the absorbance at 530 nm was determined by plate reader (Epoch, Biotech).


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