Differential roles of three regulatory proteins in IM-2/FarA signaling cascade governing secondary metabolism in *Streptomyces lavendulae* FRI-5

(放線菌 *Streptomyces lavendulae* FRI-5 の IM-2/FarA 二次代謝制御系における転写調節因子群の機能解析)

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Chapter 1

Introduction

1.1 The global threat of antimicrobial resistance and new emerging infectious diseases

Humanity has always been in constant battle with infectious diseases caused by bacteria and other pathogens. Such diseases have claimed millions of human lives and continue to threaten many more. Infectious diseases have become a global health crisis, calling for immediate strategies and action to tackle the crisis. One of the main contributing factors to the global crisis is the exponential growth of antimicrobial resistance.

Nature has blessed us with an astonishing number of secondary metabolites with important biological functions, such as the antibiotics. The study of antibiotics had a humble beginning in 1928 when Alexander Fleming fortuitously discovered the first antibiotic penicillin after he observed a clear zone encircling mold that contaminated a petri dish in his laboratory at St Mary`s Hospital in London. The impact of this discovery has been called one of the greatest contributions to medicine. Penicillin turned into a wonder drug of its time, curing thousands of individuals with bacterial infections.

Between 1940 and 1962, which is known as the golden age of antibiotics, scientists around the world discovered more than 20 novel classes of antibiotics (Fig. 1.1). The filamentous soil-inhabiting genus *Streptomyces* from the family of actinomycetes stands out as one of the major producers of clinically important antibiotics. However, bacteria and other pathogens have always found ways to survive by developing resistance to antibiotics through adaptation and mutation, creating new strains of antibiotic-resistant bacteria. If there were an endless supply of new antibiotics, antimicrobial resistance would not be a problem. Unfortunately, the rate of discovery

of new classes of antibiotics has slowed drastically since the 1960s, with only six classes of antibiotics discovered in 1963–1987 and no discoveries of new classes of antibiotics since 1987 (Silver 2011; World Economic Forum 2013) (Fig. 1.1). Thus for the almost 30 years since 1987, there has been a 'discovery void' of new antibiotics. However, in 2014 a group of scientists from the University of Notre Dame discovered a new class of non-β-lactam antibiotics (O`Daniel et al. 2014).

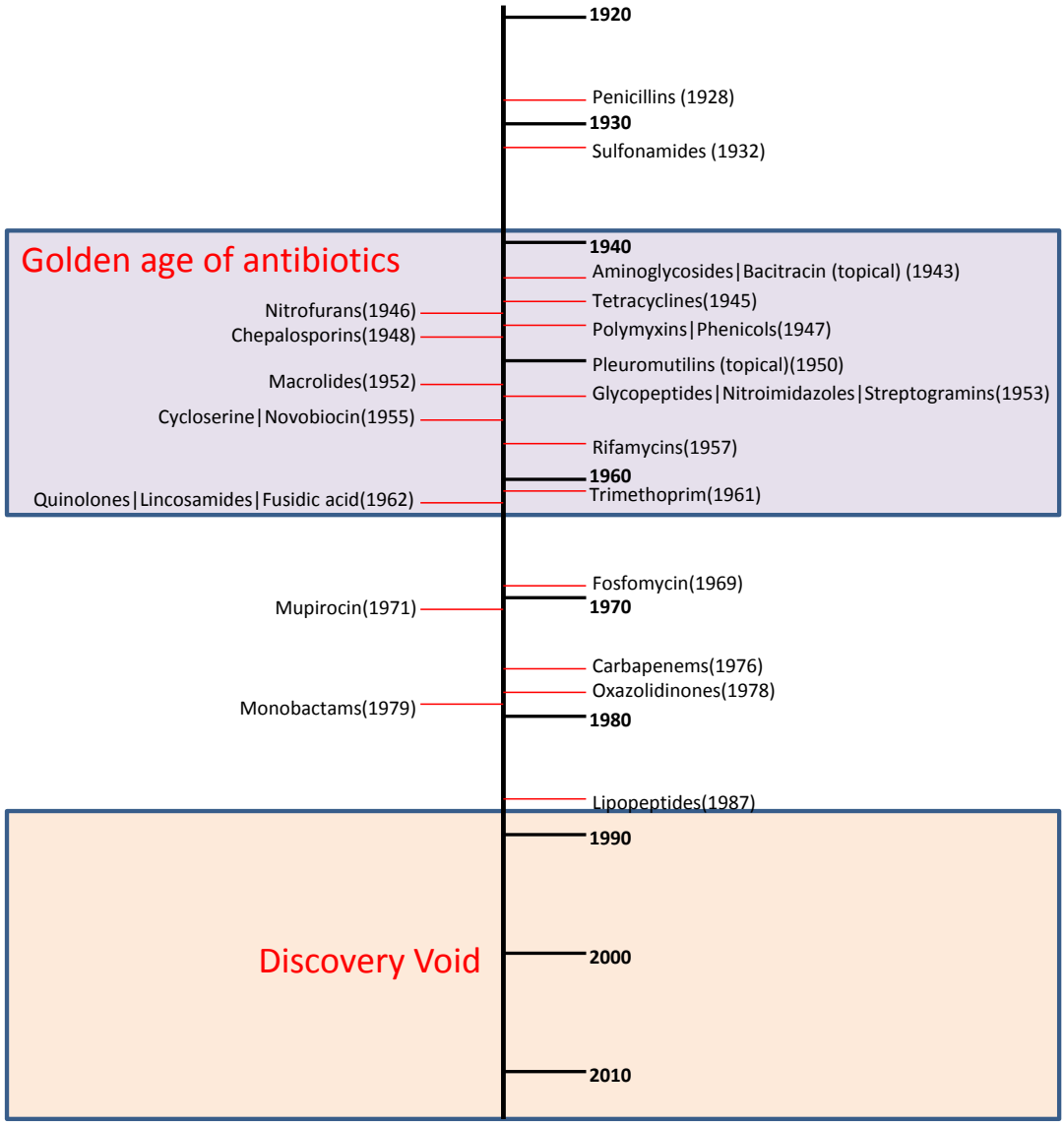


Fig. 1.1. The discovery dates of distinct classes of antibiotics. No new classes have been discovered since 1987 (Adapted from World Economic Forum 2013).

In contrast, the rate of bacteria found to have developed resistance to antibiotics is not showing any signs of slowing down and instead is aggressively increasing, sparked by the misuse and overuse of antibiotics. The more a particular antibiotic is used, the more quickly bacteria resistant to that antibiotic will be selected and then increase in number. Antimicrobial resistance is currently implicated in 700,000 deaths worldwide per year, and this number is projected to increase to 10 million deaths worldwide per year by 2050 unless action is taken (Neil 2014). Simply stated, the world is running out of antibiotics, and we are facing the threat of returning to the post-antibiotic era.

From the scientific point of view, there are several reasons why the development of new antibiotics has slowed: (1) The classical screening of new antibiotics from natural resources has proven to be laborious, costly, and ineffective as scientists have repeatedly found only known compounds over time. (2) The genome information of bacteria has revealed that the majority of biosynthetic gene clusters for secondary metabolites are silent. (3) Biosynthetic gene clusters for secondary metabolites are tightly regulated and weakly expressed, leading to the production of minute amounts of secondary metabolites which hampers the isolation and structural elucidation of the secondary metabolites. Scientists have come up with several approaches to increase the development of new antibiotics, including combinatorial chemistry to obtain new leads toward the identification of bioactive natural products, the overexpression of silent biosynthetic gene clusters in heterologous hosts to discover new compounds, and the engineering of regulatory networks controlling antibiotic biosynthesis, in order to increase the production of valuable fermentation products.

The classical screening methods were recently modified by high-throughput screening with the extensive use of combinatorial chemistry. Unfortunately, potent inhibitors (hits) are usually ineffective in the host, and the lead-to-hit ratios (<0.001%) (Berdy 2012) of synthetic libraries are

very low. Additionally, new compounds from combinatorial chemistry usually cannot be used therapeutically in light of the poor accessibility to the target (due to poor permeability or efflux). Lastly, the expensive and sophisticated instrumentation required for combinatorial chemistry means that only research groups with sufficient financial means can do the research.

The heterologous expression of biosynthetic genes cluster for secondary metabolites has been receiving greater attention in recent years due to the increasing availability of genomic information in databases and because some successful discoveries of new compounds have been made. However, the research focus is still at a premature stage due to the limited availability and compatibility of heterologous hosts (Krawczyk et al. 2013). Unlike classical screening, combinatorial chemistry, and heterologous expression approaches, engineering of the regulatory network controlling antibiotic biosynthesis offers a promising solution, based on the accumulated knowledge of the regulatory mechanisms and the availability of genetic tools. It is thus fundamentally important to gain a complete understanding of the regulatory mechanisms underlying the production of secondary metabolites.

Studies toward this end could reveal the information that is necessary for engineering regulatory networks for scientific, medical, and industrial purposes. The production of secondary metabolites is usually activated in the transition phase between primary metabolism and secondary metabolism. The regulatory mechanism is a complex biosynthetic process controlled in a hierarchical manner at different levels, with a global regulator at the highest level of the hierarchy controlling downstream low-level pathway-specific regulators that directly control the transcriptional activation or repression of biosynthetic genes for secondary metabolites.

1.2 The genus of *Streptomyces*

Actinomycetes are high-G+C Gram-positive bacteria, many of which develop a mycelial

habit. The members of the actinomycetes family produce a wide variety of secondary metabolites with various biological activities, including antibiotic, antivirus, antitumor and other pharmacologically/immunologically active substances. Among the actinomycetes, the genus of *Streptomyces* is the single most important producer of bioactive secondary metabolites, accounting for 31% of the total number of bioactive secondary metabolites identified in the 70-year period from 1940 to 2010 (Fig. 1.2) (Berdy 2012). During the golden age of antibiotics, 52% of the bioactive secondary metabolites were isolated from *Streptomyces* (Berdy 2012). Due to its medical and industrial significance, numerous scientists from all over the world have been carrying out research on all aspects of *Streptomyces* for decades, with the ultimate goal of providing the world with a continuous supply of safe and effective antibiotics as a major part of the arsenal in the war against antimicrobial resistance and new infectious diseases.

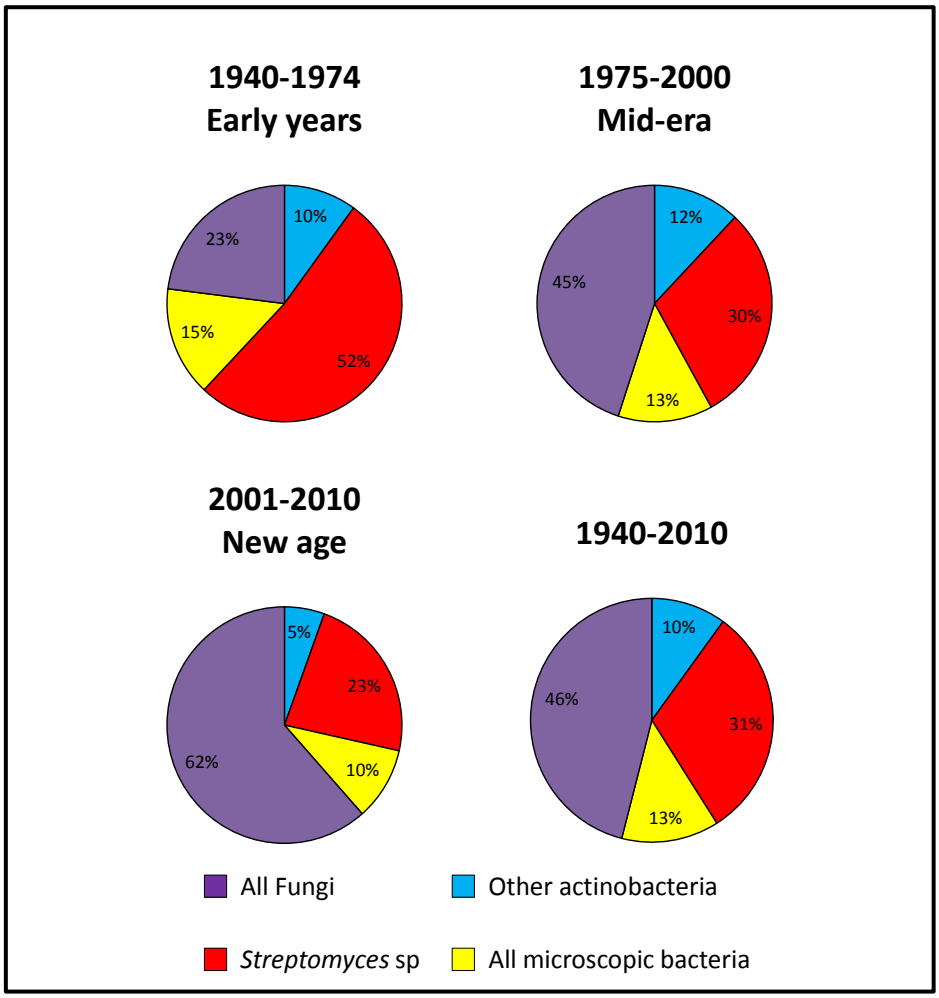


Figure 1.2 Approximate numbers of bioactive secondary metabolites in periods from 1940 to 2010 (Berdy 2012)

Streptomyces are ubiquitous in nature. Their ability to colonize the soil is greatly facilitated by their growth as a vegetative hyphal mass that can differentiate into spores that assist in the spread and persistence of these bacteria. The life cycle of *Streptomyces* begins with the growth of a colony from a spore, which under suitable conditions triggers the germination of tubes emerging from the unigenomic spore. The tubes grow by tip extension and branch formation to give rise to the formation of a substrate mycelium (Fig 1.3)(Kieser et al. 2000). In response to nutrient limitation and other types of physiological stress, *Streptomyces* undergo further changes

including the onset of secondary metabolism, the lysis of some compartments of the substrate mycelium (thus releasing nutrients), and the initiation of aerial hyphal growth. The aerial hyphae growth further, forming a spiral synctitium that contains many tens of genomes. When the aerial hyphae growth stops, the aerial hyphae subdivides into a unigenomic pre-spores compartment, followed by the thickening of walls and the deposition of a gray spore pigment to generate dessicant-resistant spores.

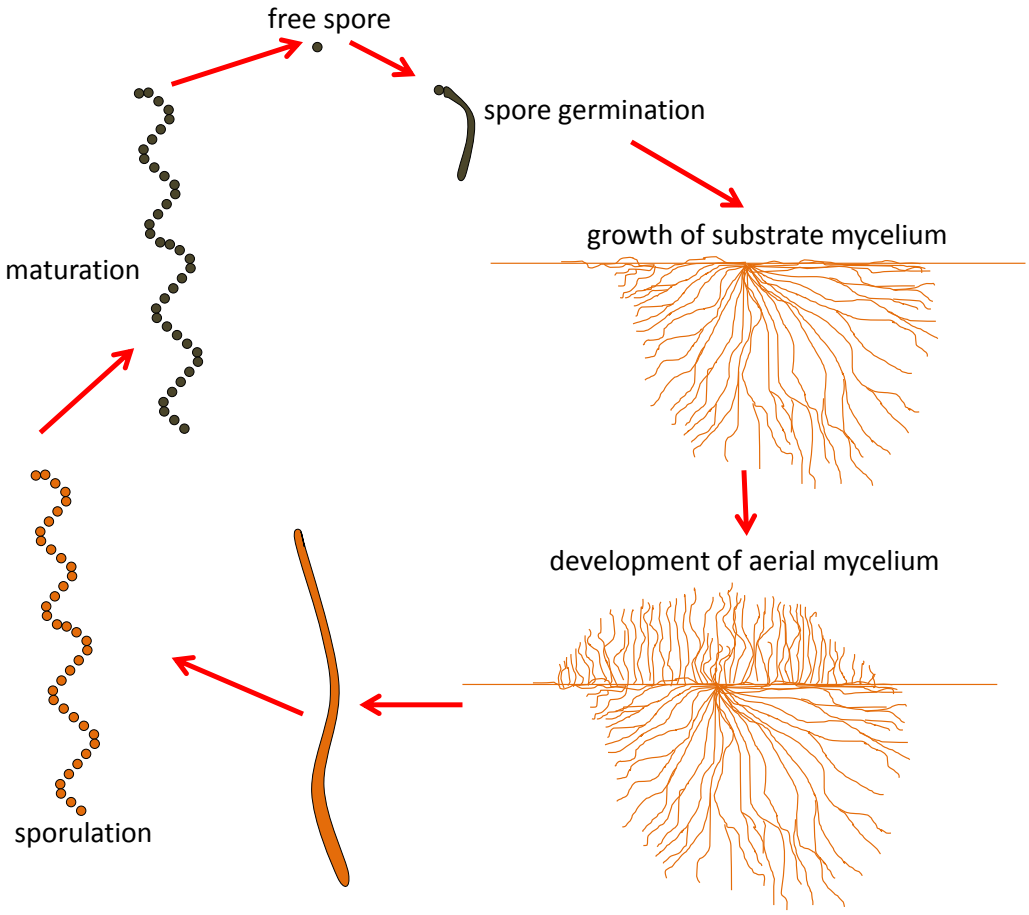


Figure. 1.3 The life cycle of *Streptomyces*. (adapted from lisci.kitasato-u.ac.jp)

In regard to the secondary metabolism, research on *Streptomyces* has focused mainly on two approaches: the phenotypic approach and the genetics approach. The phenotypic approach includes the classical screening method that uses soil *Streptomyces*, which is still regarded by

many as a promising technique in the search for new or novel derivatives of bioactive secondary metabolites. However, the phenotypic approach requires laborious effort for screening, and it has resulted in the repeated re-discovery of known compounds. In addition, many researchers have shifted their focus to a different source of *Streptomyces*, i.e., marine environments. Marine *Streptomyces* live in habitats that are completely different from those of soil *Streptomyces*, which subjects them to different types of physiological stress compared to soil *Streptomyces*. The different living conditions of marine *Streptomyces* are speculated to contribute to the production of secondary metabolites with novel features. There have been a few successful discoveries of new bioactive secondary metabolites from the marine *Streptomyces* (Igarashi et al. 2010; Abdelmohsen et al. 2010)

The genetics approach is rapidly gaining in popularity, especially since the dawn of the genomic era, which began with the publication of the first completed genome sequence of the model *Streptomyces coelicolor* A3(2) in 2002 (Bentley et al. 2002). Since then, numerous *Streptomyces* genomes have been sequenced and deposited in the databases. *Streptomyces* genomes have indicated that *Streptomyces* are still a promising source in the search for new bioactive secondary metabolites. It was revealed that *Streptomyces* possess the coding capacity to produce multiple secondary metabolites with as few as 20 biosynthetic gene clusters for secondary metabolites were found in the genome, the majority of which are silent under laboratory conditions.

The capacity to produce multiple secondary metabolites might also reflect the selective use of particular secondary metabolite(s) depending on the primary metabolic flux and the availability of precursors, or a combination of these. It was also revealed that multiple regulatory genes are spread throughout the genome, confirming the complex regulatory networks for secondary metabolism in *Streptomyces*. In general, research on the genetics of *Streptomyces* can

be narrowed down to a few themes: (i) characterization of the regulatory mechanism for the production of secondary metabolites; (ii) the engineering of secondary metabolite pathways, including activation of the silent biosynthetic gene cluster and the heterologous expression of the biosynthetic gene cluster; and (iii) the engineering of regulatory cascades and networks controlling secondary metabolite production.

1.3 The regulatory networks controlling secondary metabolism in *Streptomyces*

The majority of the bioactive secondary metabolites produced by *Streptomyces* are toxic by nature, and their production must therefore be regulated to ensure the exact timing and quantity of production. The natural function of many *Streptomyces* secondary metabolites is thought to be antibiotics that thwart the growth of competing microorganisms. In the event of nutrient depletion or other types of physiological stress, *Streptomyces* undergoes a complex morphological differentiation, producing spores at the ends of aerial hyphae that grow out from the vegetative mycelial mass. The sporulation process is fueled by nutrients released from a portion of the mycelia. At this stage of differentiation, secondary metabolites such as antibiotics are produced as a defense mechanism and barrier against the scavenging activity of the competing organism.

The timing and quantity of secondary metabolite production is strictly regulated in a complex manner, implicating numerous regulatory proteins forming regulatory networks. The regulatory networks for secondary metabolites are often organized in a hierarchical manner, in which high-level regulatory proteins modulate the expression of downstream low-level pathway-specific regulatory genes that directly control the transcriptional activation and repression of biosynthetic genes for secondary metabolites.

The high-level regulators are often regarded as global regulators that convert stimuli signals from the environment into cellular processes. Genes that encode global regulators are most

commonly located outside of the biosynthetic gene clusters for secondary metabolites, and they exert pleiotropic effects on the production of secondary metabolites and/or morphological differentiation. In *Streptomyces*, the most extensively studied high-level regulators are those belonging to the group of γ-butyrolactone autoregulators and their cognate receptor proteins (Takano 2006). Other high-level regulators belong to a group of bacterial two-component systems (TCSs) that respond to a variety of external stress signals (e.g., phosphate, carbon or nitrogen starvation). There are a large number of TCSs in *Streptomyces*. One of the best-studied is the phosphate control of *Streptomyces* metabolism by the PhoR-PhoP TCS (Martin 2004). Another important system in the control of secondary metabolite biosynthesis in *Streptomyces* is the nitrogen-source regulation mediated by the orphan response regulator GlnR (Wray et al. 1991 & 1993).

The low-level regulators, which are frequently regulated by global regulators, are usually transcriptional activators of a biosynthetic gene cluster for the secondary metabolite. Several families of these regulators exist, including the *Streptomyces* antibiotic regulatory protein (SARP) family (Arias et al. 1999), the StrR family (characterized by a helix-turn-helix DNA-binding motif) (Retzlaff et al. 1995), and the large regulators of the LAL-family (Anton et al. 2004). Genes encoding low-level regulators are usually found clustered together with biosynthetic genes for the transcription they regulate.

The isolation and structure elucidation of secondary metabolites produced by microorganisms are often hampered due to tight regulation and low productivity. Therefore, the elucidation of regulatory mechanisms controlling the production of secondary metabolites is fundamental, as such studies could obtain the information necessary to overcome the difficulties of the isolation process and low productivity. In fact, studies of the regulation of secondary metabolite production have provided (Viet et al. 2007; Malla et al. 2010) and will continue to

provide new strategies for "awakening" the production of secondary metabolites such as antibiotics, thereby allowing us to explore more effectively the rich stores of natural products encoded in the genome of *Streptomyces*. In *Streptomyces*, the regulation mechanism is often mediated by small signaling molecules, with its cognate receptor forming a regulatory cascade that converts signals from the environment into cellular processes.

1.4 Signaling molecules in *Streptomyces*

The signaling molecules in *Streptomyces* are low-molecular-weight "bacterial hormones" (also called autoregulators) that are essential for the regulation of secondary metabolites' biosynthesis and/or morphological differentiation. Based on their chemical structure, the signaling molecules are classified into three types: (i) the γ-butyrolactone type, (ii) the furan type (Corre et al. 2008), and (iii) the recently identified butenolide type (Kitani et al. 2011). The signaling molecules modulate the secondary metabolites' biosynthesis and/or morphological differentiation by binding with the cognate cytoplasmic receptor protein. The signaling molecules' cognate receptors are usually repressors of secondary metabolism. The binding of the signaling molecules with the cognate receptor releases the repression activity of the receptor, and thus in general the signaling molecules act as activators. In *Streptomyces*, one particular type of widely distributed signaling molecule that has drawn a significant amount of attention due to its close association with antibiotic production is the γ-butyrolactone autoregulators.

1.5 The γ-butyrolactone autoregulators and their cognate receptors

The structures of 14 γ-butyrolactones autoregulators isolated from seven *Streptomyces* species have been determined as of this writing (Fig. 1.4) (Takano 2006). The structures are similar in that they have a 2,3-disubstituted butyrolactone skeleton in common. The structures

differ in length, branching, and the stereochemistry of their fatty acid side-chain (Takano 2002). The chemical structure of γ-butyrolactones is similar to that of N-acyl-homoserine lactones (AHLs) in proteobacteria except for the carbon side chain. However, due to the low similarity of these signaling molecule receptors, the γ-butyrolactone receptors do not bind to AHLs and vice versa. AHLs and γ-butyrolactones seem to have different functions, as AHLs show diverse properties whereas γ-butyrolactones mainly regulate the secondary metabolite production and morphological differentiation.

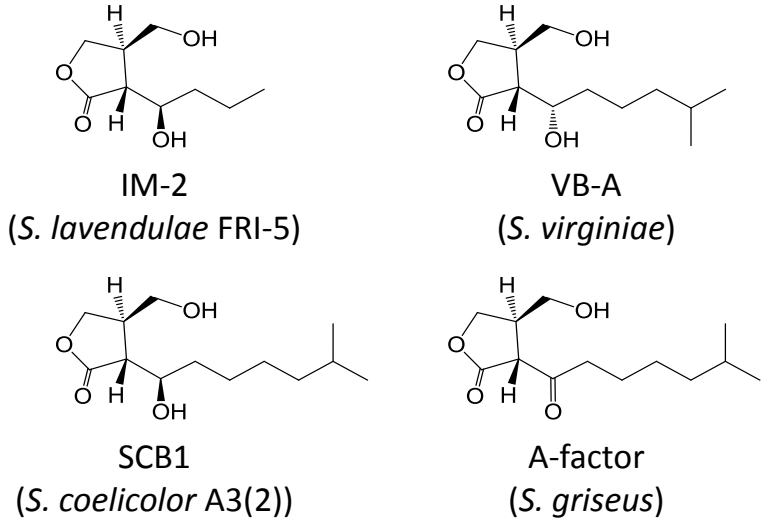


Figure 1.4 Structures of γ-butyrolactone autoregulators from *Streptomyces.*

Based on the stereochemistry of their fatty acid side chain, γ-butyrolactones can be classified further into the following three groups: (i) the Virginiae butanolide (VB) type, with a 6-alpha-hydroxyl group, to which VBA–VBE of *S. virginiae* and Grafe's three factors belong; (ii) the A-factor type, with a 6-keto group, to which only the A-factor of *S. griseus* belongs; and (iii) the IM-2 type, with a 6-beta-hydroxyl group, to which IM-2 of *S. lavendulae* FRI-5, SCB1 of *S. coelicolor*, and factor I belong. All of these factors regulate the production of secondary

metabolites; they are effective at nanomolar concentrations, and in some cases (such as in *S. griseus*), they regulate morphological differentiation. They are widely distributed in other *Streptomyces* species. Hashimoto et al. (1991) reported that at least 30-40 % of *Streptomyces* species would produce one of those autoregulators.

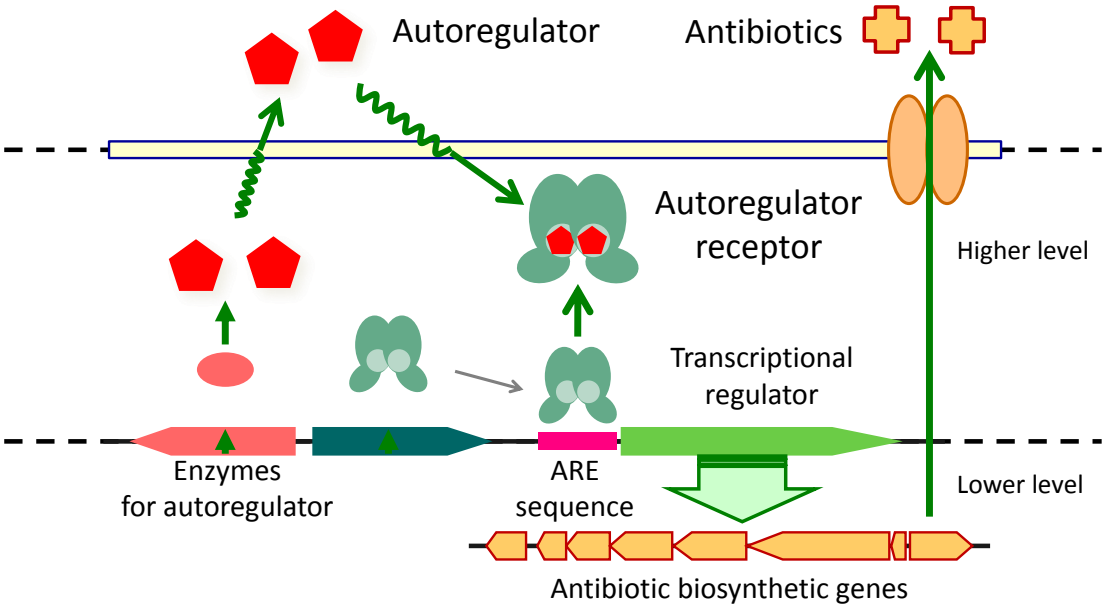


Figure. 1.5 The molecular mechanism of an autoregulator signaling cascade.

The γ-butyrolactone autoregulators bind specifically to cognate cytoplasmic receptor proteins and inhibit their binding to specific DNA targets (Fig. 1.5). Specific DNA targets are usually located in the promoter region of target genes, and therefore most of these receptor proteins act as transcriptional repressors by blocking the access of RNA polymerase to the promoter region to initiate transcription. Thus, the binding of receptor proteins to their cognate γ-butyrolactone autoregulators induces the transcription of target genes. Target genes of the γ-butyrolactone receptor proteins are usually pathway-specific transcriptional regulators that act at the lowest level of the regulatory cascade for secondary metabolism.

Table 1.1 Distribution of signaling molecules and their cognate receptors in *Streptomyces*

Species	**Receptor**	**Antibiotics**	**Signaling molecule**
S. virginiae	*barA*	Virginiamycins	VBs
S. lavendulae	*farA*	D-cycloserine Blue pigment Showdomycin	IM-2
S. coelicolor	*scbR*	Actinorhodin Undecylprodigiosin	SCB1, SCB2, SCB3
S. natalensis	*sngR*	Natamycin	ND
S. scabies NBRC12914	*sscR*	ND	ND
S. avermitilis	*avaR3* *avaL1*	Avermectin ND	Avenolide ND
S. griseus	*arpA*	Streptomycin	A-factor
S. fradiae	*tylP*	Tylosin	ND
S. ambofaciens	*alpZ*	Alpomycin	ND
S. clavuligerus	*scaR* (*brp*)	Clavam	ND

ND: not determined.

1.5.1 The A-factor signaling cascade

The first γ-butyrolactone molecule and also the best characterized, the A-factor, was isolated and identified in 1967 from *Streptomyces griseus* by a group of Russian scientists. A-factor is important in the stimulation of morphological differentiation and the production of the secondary metabolite streptomycin. In 1995, a Japanese research group led by Dr. Sueharu Horinouchi identified the A-factor receptor protein ArpA (Kudo et al. 1995) and the biosynthetic gene for the synthesis of A-factor (Kato et al. 2007), as well as the A-factor signaling cascade. In the absence of A-factor, ArpA protein binds to a specific sequence in the upstream region of a global regulatory gene, *adpA*. When A-factor is produced and reaches a critical threshold concentration, ArpA dissociates from the upstream region of *adpA* by binding with A-factor, thereby triggering the transcriptional activation of *adpA*. AdpA then activates the transcription of many genes required for secondary metabolism and morphological differentiation, including *strR*, which is the pathway-specific regulator for the streptomycin biosynthetic genes, and *amfR*, which is important for aerial mycelium formation.

The A-factor signaling cascade follows the concept of a hierarchical regulatory mechanism, with A-factor and its cognate receptor ArpA situated at the highest level of the signaling cascade. The A-factor is the only γ-butyrolactone known to be involved in the developmental process of a *Streptomyces* species.

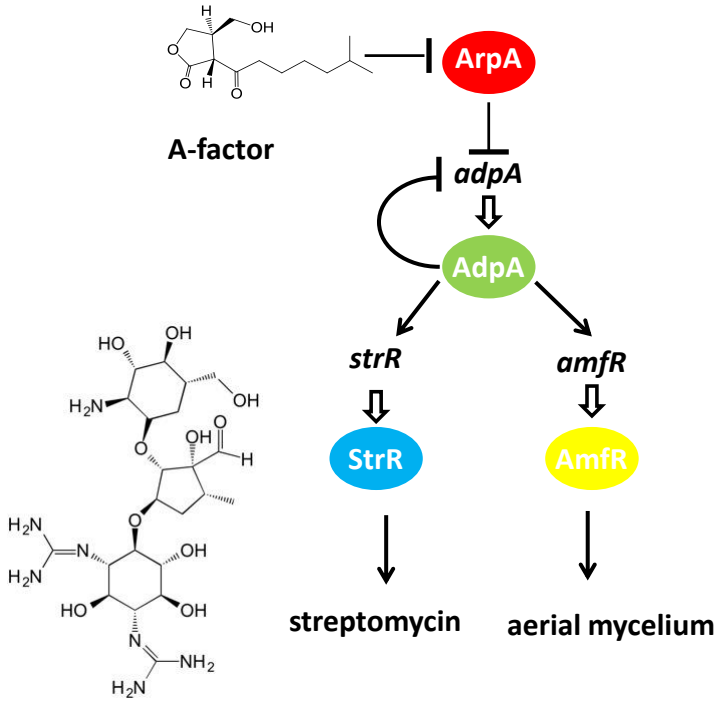


Figure. 1.6 The A-factor signaling cascade in *Streptomyces griseus*.

1.5.2The Virginiae Butanolides (VB) signaling cascade

In *S. virginiae*, the γ-butyrolactone virginiae butanolides (VB) and the cognate receptor BarA regulate the production of the virginiamycins VM and VS (Kim et al 1989, 1990; Nihira et al. 1988). Two genes encoding a *barA* homologue are present in the vicinity of the *barA* gene. One *barA* homologue gene, *barB*, is under the transcriptional control of the receptor BarA. BarB represses the expression of the remaining *barA* homologue *barZ* and a gene (*vmsR*) that belongs to the SARP family. VmsR acts as an activator of the biosynthesis of the virginiamycins VM and VS through the transcriptional activation of two pathway-specific regulatory genes, *vmsS* and *vmsT* (Pulsawat et al. 2009). VmsS, which belongs to the SARP family, is necessary for the production of both VM and VS. For the production of VM, only VmsT (which showed similarity to a response regulator of a bacterial two-component signal transduction system that appears to lack a cognate sensor kinase) is necessary.

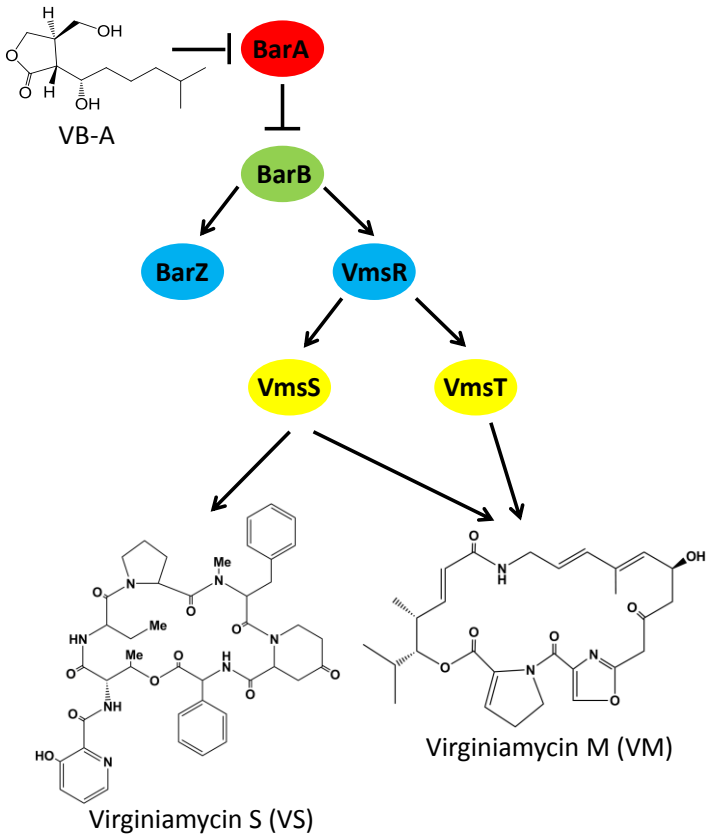


Figure 1.7 The VB signaling cascade in *Streptomyces virginiae*.

1.5.3 The SCB1 signaling cascade

The IM-2 type γ-butyrolactone autoregulator of *S. coelicolor*, SCB1, acts to regulate the production of actinorhodin and undecylprodigiosin, the two pigmented antibiotics from *S. coelicolor* (Takano et al. 2001). Similar to other γ-butyrolactone autoregulators, SCB1 binds to its cognate receptor ScbR. Before SCB1 production, the ligand-free ScbR represses the transcription of its own gene and possibly that of *scbA*, an *afsA* homologue gene important for the biosynthesis of γ-butyrolactones. Under these conditions, ScbR represses the synthesis of an unidentified negative regulator for antibiotic production (RSM).

ScbA is required for SCB1 production and also for the transcriptional activation of *scbA* by binding with ScbR, forming the ScbRA complex. In the transition phase, ScbA is accumulated to

a level sufficient to form a complex with ScbR. The ScbRA complex activates *scbA* transcription, which leads to a burst of SCB1 production. When SCB1 reaches the threshold concentration, it binds to ScbR, relieving self-repression and inactivating the ScbRA complex, which leads to a reduction in *scbA* transcription. This leads to a decline in SCB1 levels, which, coupled with the remaining high levels of ScbR, leads to the repression of RSM, thereby triggering the production of two pigmented antibiotics.

1.5.4 The IM-2/FarA signaling cascade

Among the known γ-butyrolactone autoregulator signaling systems, the IM-2 system with its cognate receptor FarA in *S. lavendulae* FRI-5 possesses unique characteristics with respect to the control of secondary metabolism. To date, IM-2 is the sole γ-butyrolactone autoregulator that serves not only as an inducer but also as a repressor of secondary metabolite production. IM-2 triggers the production of blue pigment and the nucleoside antibiotics showdomycin and minimycin, but it represses the production of the antituberculosis antibiotic D-cycloserine, indicating that the IM-2/FarA signaling cascade employs a more sophisticated regulatory mechanism to control secondary metabolism.

1.5.5 A brief history of the IM-2 signaling cascade

Our research group has had a long history (over 25 years) of efforts to unravel the IM-2/FarA signaling cascade (Fig. 1.11). It began in 1988 with the discovery of an inducing molecule designated as IM-2 that can induce blue pigment production in *Streptomyces lavendulae* FRI-5 at the concentration of 0.6 ng/mL (Yanagimoto et al. 1988). IM-2 production can be divided into three phases, pre-activation, activation, and post-activation. In the pre-activation phase, IM-2 is not produced. In activation phase, IM-2 is started to produce at the

beginning of exponential phase and usually reaches a threshold concentration in the mid exponential phase (8h) and triggers blue pigment production roughly 2 hr later (Fig. 1.8). A bioassay-based IM-2 assay showed that in the post-activation phase, the IM-2 concentration rapidly decreased over time to a negligible level just shortly after reaching the threshold concentration (Yanagimoto et al. 1988).

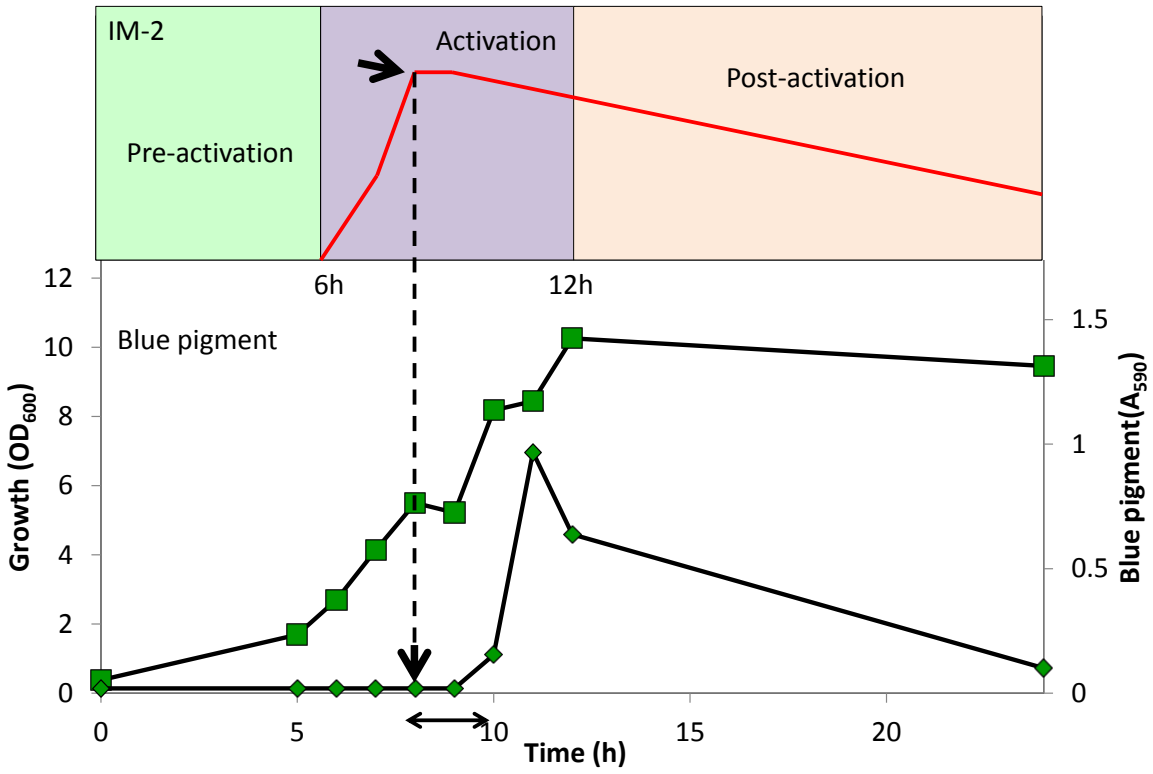


Figure. 1.8 Growth curves and indigoidine production in the wild-type strain. Growth was monitored by measuring the optical density at 600 nm (OD_{600}). Green filled circles indicate growth curves. Green diamonds indicate production profiles of blue pigment.

The structure of IM-2 was found to be (2R,3R,1'R)-2-1'-hydroxybutyl-3-hydroxymethyl-γ-butanolide (Sato et al. 1989). IM-2 was also observed to induce the production of the nucleoside antibiotics showdomycin and minimycin, but it represses D-cycloserine production (Hashimoto et al. 1992).

A few years after the identification of IM-2, the IM-2 receptor protein designated as FarA (FRI-5 BarA homolog) was identified and purified, followed by the identification of the gene encoding the receptor protein and a putative biosynthetic gene for IM-2 biosynthesis, *farX* (Waki et al. 1997). FarA was found to act as a transcriptional repressor of *farA* itself, forming an autoregulatory circuit, and a gene replacement analysis revealed that FarA acts as a regulator in the secondary metabolism of *S. lavendulae* FRI-5 (Kitani et al. 1999, 2001). FarA positively regulates the biosynthesis of IM-2 and negatively regulates the biosynthesis of the blue pigment and nucleoside antibiotics (Fig. 1.9A). The intact FarA and the presence of IM-2 are necessary for the termination of D-cycloserine production, suggesting that the IM-2/FarA complex may have regulatory roles (Fig. 1.9B).

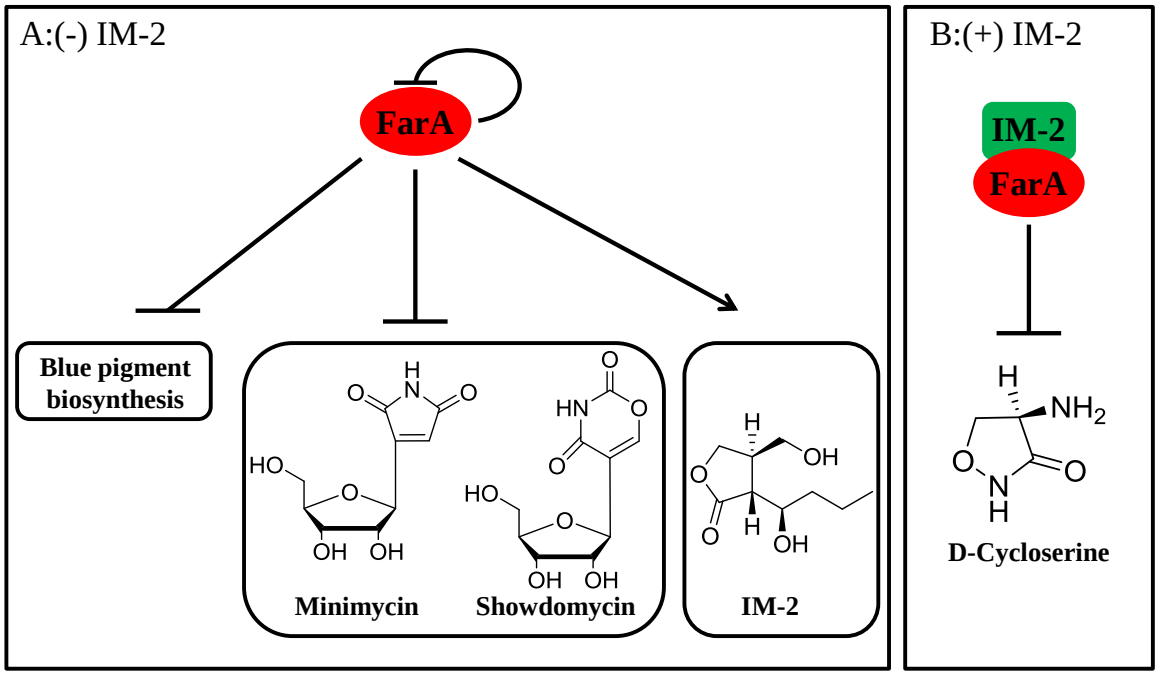
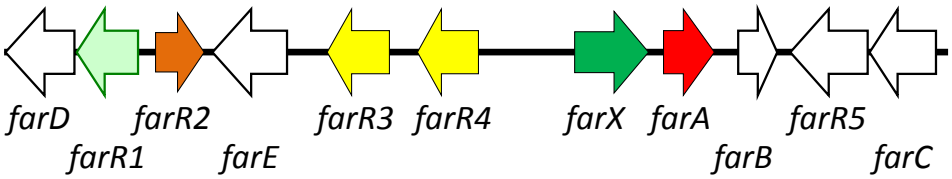


Figure 1.9 The two modes of the IM-2 signaling cascade in *Streptomyces lavendulae* FRI-5. A: In the absence of IM-2. B: In the presence of IM-2.

Multiple putative regulatory genes were then found in the flanking region of *farA*, forming a regulatory island designated as a *far*-regulatory island (Fig. 1.10). In addition to *farA* and the putative IM-2 biosynthetic gene *farX*, there are five more putative regulatory genes, named *farR1* to *farR5* (Kitani et al. 2008). *farR1* encodes a homolog of response regulators of bacterial two-component signal-transduction systems that appear to lack a cognate sensor kinase. *farR2* is highly similar to the TetR family of transcriptional regulators, and it most closely resembles the γ-butyrolactone receptor. Two genes, *farR3* and *farR4*, are homologous to the SARP family members, which usually act as pathway-specific transcriptional activators. *farR5* shows similarity to transcriptional activators in the AraC family.



Gene	Proposed function
farD	Unknown
farR1	Response regulator
farR2	**γ-butyrolactone receptor homologue**
farE	Cyclase, aromatase
farR3	**SARP-family regulator**
farR4	**SARP-family regulator**
farX	IM-2 biosynthetic enzyme
farA	IM-2 receptor
farB	Acetyltransferase
farR5	AraC-type transcriptional regulator

farC	Epimerase

Figure 1.10 Gene organization of the *far*-regulatory island and the proposed function of each gene.

In 2010, we confirmed that *farX*, an *afsA* homologue, is essential as a biosynthetic gene for IM-2 production, and we demonstrated that IM-2 controls the transcription of *farX*, indicating a negative autoregulatory circuit for the production of γ-butyrolactone (Kitani et al. 2010). Taken together with the positive regulation of FarA in IM-2 biosynthesis, these findings suggested that *S. lavendulae* FRI-5 has a fine-tuning system to control γ-butyrolactone biosynthesis. Our data suggested that IM-2/FarA is situated at the highest level in the signaling cascade controlling the biosynthesis of blue pigment, showdomycin, minimycin and D-cycloserine, probably by regulating the transcriptions of low-level pathway-specific regulatory genes in the *far*-regulatory island.

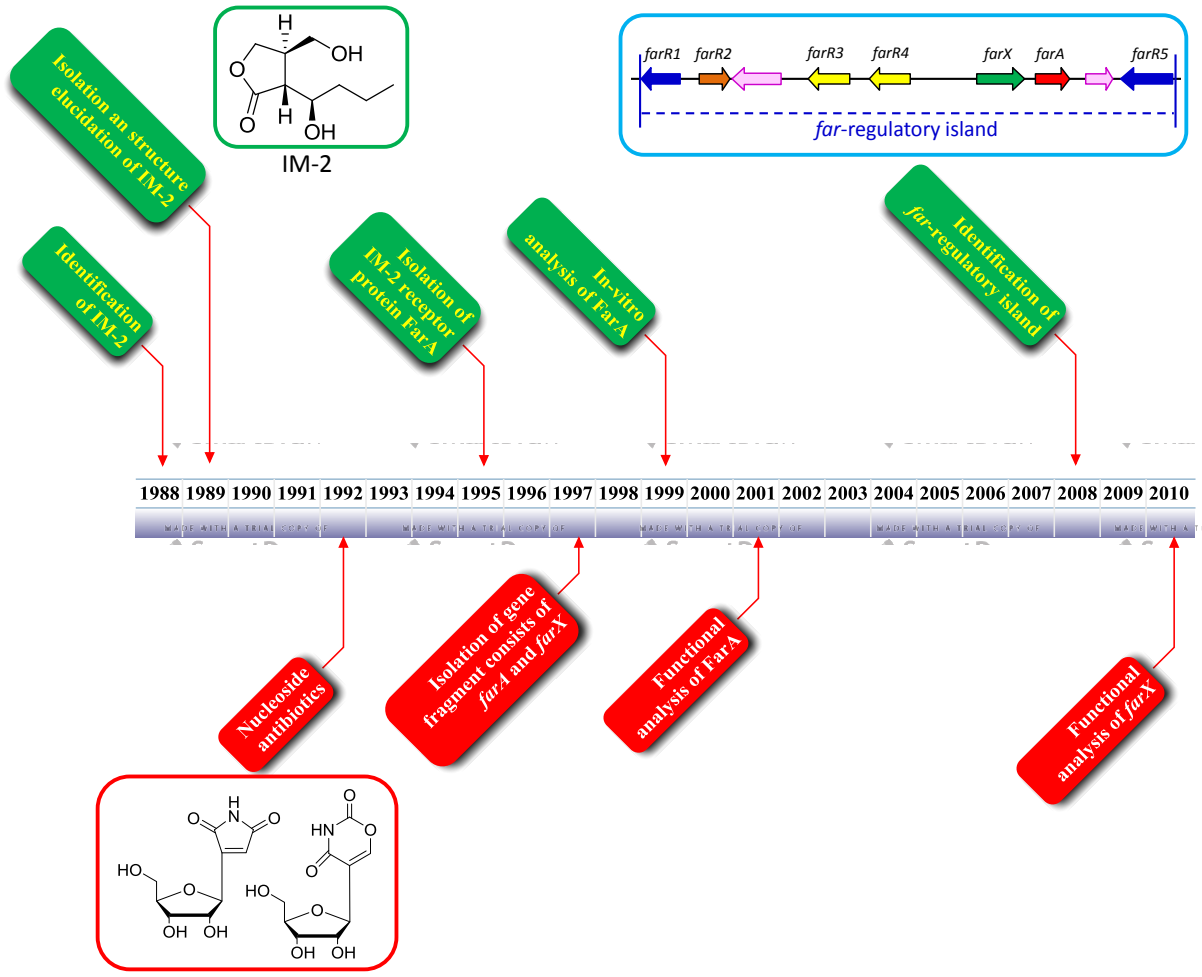


Figure 1.11 Timeline of the research to clarify the IM-2 signaling cascade in *S. lavendulae* FRI-5.

1.6 The aim of the present study

As described in previous sections, the γ-butyrolactone signaling cascade in *S. lavendulae* FRI-5 is unique, complex, and largely still poorly understood. The functions of the receptor protein FarA and IM-2 have been extensively studied but provide no direct link with regard to the production of the secondary metabolites of *S. lavendulae* FRI-5, suggesting that several layers of regulation are present in the IM-2/FarA system. The IM-2/FarA system is situated at the highest level in the signaling cascade, and thus IM-2/FarA should elicit its function through the transcriptional regulation of low-level pathway-specific regulators.

Our previous studies have shown two different roles of FarA in the transcriptional regulation of target genes. First, following the general molecular mechanism of autoregulator signaling cascades, the transcription of target genes is repressed through the binding of FarA to FAREs (FarA responsive element). The binding of IM-2 to FarA results in the dissociation of FarA from FAREs and allows the transcriptional activation of target genes. Secondly, the IM2/FarA complex itself has a DNA binding activity for controlling the expression of the target genes.

Regulatory genes are usually located in the vicinity or in the middle of biosynthetic genes for secondary metabolites, and occasionally they form a regulatory island. This is the case with *S. lavendulae* FRI-5, in which we found five putative regulatory genes (*farR1–farR5*) in the flanking region of *farA–farX*, forming the *far*-regulatory island. The four genes *farR1–R4* come from three different protein families which have been closely associated with the butyrolactone signaling cascade in other *Streptomyces* species. An in vitro binding assay and S1 nuclease mapping revealed that at least three regulatory genes, *farR1*, *farR2*, and *farR4*, are under the transcriptional control of FarA, suggesting that they may play important roles in the IM-2 signaling cascade.

Thus, a further understanding of what roles the regulatory genes in the *far*-regulatory island might play will provide greater insights into how the IM-2/FarA signaling cascade is activated and terminated to control secondary metabolism in *S. lavendulae* FRI-5.The objective of my study was thus to characterize the regulatory genes in the *far*-regulatory island that are probably involved in the IM-2 signaling cascade. An overview of the study is as follows.

Chapter 2 describes the characterization of two genes, *farR3* and *farR4*, tandemly located in the flanking region of *farA*. To investigate the functions of *farR3* and *farR4*, I constructed three deletion mutants (i.e., the double disruptant *farR3R4*, the *farR3* disruptant, and the *farR4*

disruptant) and assessed them for the production of blue pigment and IM-2. The results confirmed that FarR3 is a typical pathway-specific regulator that activates blue pigment production, and this regulator was later identified as indigoidine. FarR4 was found to be essential as a negative regulator of IM-2 biosynthesis, and this is therefore the first report that a SARP family regulator modulates the biosynthesis of a γ-butyrolactone molecule.

Chapter 3 describes the characterization of *farR2*, a γ-butyrolactone receptor homolog gene. I performed in-frame gene deletion and complementation analyses to clarify its in vivo function. The results confirmed that FarR2 makes two different contributions to the signaling cascade: as an activator of the production of the blue pigment indigoidine and as a repressor important to the termination of IM-2 and indigoidine production.

Lastly, in Chapter 4 I present a new working model of the IM-2/FarA signaling cascade that functions to control IM-2 and indigoidine production, as a general summary and conclusions.

Chapter 2

Differential contributions of two SARP family-regulatory genes to blue-pigment indigoidine biosynthesis in *Streptomyces lavendulae* FRI-5

2.1 Introduction

Members of the Gram-positive, soil-dwelling filamentous bacterial genus *Streptomyces* have been extensively studied due to their complex life cycle of morphological differentiation and their ability to synthesize antibiotics of structural and biological diversity possessing medical and industrial significance. The regulation of secondary metabolism in *Streptomyces* is a complex process controlled in a hierarchical manner at different levels: higher-level regulators, regarded as global regulators that transmit signals from the environment, activate lower-level pathway-specific regulators which directly control the expression of gene clusters responsible for the biosynthesis of individual secondary metabolite(s) (Bibb 2005; Liras et al. 2008). Higher-level regulatory genes are most commonly localized outside of biosynthetic gene clusters, and exert pleiotropic effects on both secondary metabolism and morphological development, or influence the production of multiple secondary metabolites but do not affect morphological development. On the other hand, lower-level pathway-specific regulators act as a master switch for the biosynthesis of a single secondary metabolite, and these genes are usually found within the respective biosynthetic gene cluster. Among the pathway-specific regulators, the *Streptomyces* antibiotic regulatory protein (SARP) family regulators are well-studied (Wietzorrek and Bibb 1997) and frequently used for deciphering the regulatory mechanisms of secondary metabolite production (Pulsawat et al. 2009; Tanaka et al. 2007). The SARP-family regulators are DNA-binding proteins which share sequence similarities with members of the OmpR family DNA-

binding proteins. In *Streptomyces coelicolor* A3(2), the SARP-family regulators control the production of both actinorhodin (ActII-ORF4) and undecylprodigiosin (RedD) (Arias et al. 1999; Takano et al. 1992). Because the number of SARP-family proteins registered to various databases is continuously increasing, knowledge of the regulation mechanisms by the SARP-family regulators is of great interest, and could potentially allow an increase in the yields of secondary metabolites in the producer strains.

Streptomyces lavendulae FRI-5 produces blue pigment, nucleoside antibiotics, and the antituberculosis antibiotic D-cycloserine as secondary metabolites. The production of these secondary metabolites is controlled by IM-2, one of the small diffusible signaling molecules called "γ-butyrolactones" (sometimes, "γ-butyrolactone autoregulators") (Hashimoto et al. 1992). Most of the γ-butyrolactones play a positive role in the regulation of secondary metabolite production, such as the production of streptomycin in *Streptomyces griseus* (Horinouchi 2007), the production of actinorhodin and undecylprodigiosin in *Streptomyces coelicolor* A3(2) (Takano et al. 2000), and the production of virginiamycin in *Streptomyces virginiae* (Yamada et al. 1987). In contrast to the solely positive effects exerted by other autoregulators, IM-2 has opposing effects on the regulation of secondary metabolism; namely, it not only switches on the production of blue pigment and nucleoside antibiotics, but also switches off the production of D-cycloserine (Hashimoto et al. 1992). IM-2 production is initiated before the mid-exponential phase, and the IM-2 concentration reaches a threshold at the mid-exponential phase, resulting in the onset of the secondary metabolism 1.5-2 h later (late-exponential phase) (Yanagimoto et al. 1988). After entry into the stationary phase, IM-2 production declined gradually in a growth-dependent manner. The IM-2-specific receptor (FarA), which exhibits DNA-binding activity toward FAREs (FarA-responsive elements), plays a critical role in the biosynthetic regulation of the metabolites, including IM-2 itself (Kitani et al. 1999, 2008). FarA negatively controls the biosynthesis of blue

pigment and nucleoside antibiotics, and the IM-2-FarA complex is postulated to be the essential component in the termination of D-cycloserine production (Kitani et al. 2001). By contrast, FarA also has another function as a positive regulator of the biosynthesis of IM-2 (Kitani et al. 2001). Thus, FarA could be regarded as a higher-level regulator in the IM-2 signalling cascade.

In *Streptomyces* species, multiple regulatory genes, directly or indirectly controlling the biosynthesis of a particular secondary metabolite(s), are frequently localized in the vicinity or in the middle of the biosynthetic gene cluster, and they occasionally form a regulatory island (Aigle et al. 2005; Pulsawat et al. 2007). We previously found that the *farA*-flanking region has seven regulatory genes, including *farX*, an IM-2 biosynthetic gene, and comprises a *far* regulatory island (Kitani et al. 2008, 2010). Two putative regulatory genes (*farR3* and *farR4*) encoding the SARP-family protein are present in the *far* regulatory island together with two more putative transcriptional regulatory genes (*farR1* and *farR2*) (Fig. 2.2a), all of which are considered to be the direct transcriptional targets of FarA. The transcription of *farX* starts at the early exponential phase, and increases gradually to initiate IM-2 biosynthesis. When IM-2 concentration reaches a critical level at the mid-exponential phase, IM-2 binds to FarA sitting on FAREs (FARE2 and FARE3, in the promoter of *farR1* and *farR2*) and the IM-2-FarA complex dissociates from the FAREs, resulting in upregulation of transcriptions of *farR1* and *farR2* and the production of secondary metabolites including blue pigment at the late exponential phase. We previously found that FARE4 in the upstream region of *farR4* is the FarA-binding site, and a FARE-like sequence is present in the upstream region of *farR3* (Kitani et al. 2008). Phenotypic analysis of the IM-2-deficient strain showed that the transcriptional upregulation of *farR3* and *farR4* at the late exponential phase is eliminated by gene deletion of *farX*, and thus the *farR3* and *farR4* genes are members of the IM-2 stimulon, although the direct transcriptional control of the two SARP-family proteins by FarA remains unclear (Kitani et al. 2010). These findings led me to investigate

the roles played by *farR3* and *farR4* in the regulation of secondary metabolite production in *S. lavendulae* FRI-5. In this study, I characterized the functions of *farR3* and *farR4* in the IM-2/FarA signalling cascade for secondary metabolism, and demonstrated that FarR3 positively controls the production of blue pigment indigoidine and FarR4 is negatively involved in the biosynthesis of IM-2, implying that the lower-level SARP-family regulators contributes differentially to the pyramidal cascade governing secondary metabolism.

2.2 Materials and Methods

2.2.1 Bacterial strains, plasmids, and growth conditions

Streptomyces lavendulae FRI-5 (MAFF10-06015; National Food Research Institute, Tsukuba, Japan) was grown on ISP medium 2 (Becton, Dickinson and Company, Franklin Lakes, NJ) for spore formation. *Escherichia coli* DH5*α* was used for routine cloning procedures (Sambrook and Russel 2001) and the DNA methylation-deficient *E. coli* strain ET12567 containing pUZ8002 (Paget et al. 1999) was used for *E. coli/Streptomyces* conjugation. The plasmids used were pBluescript II SK for general cloning, pKC1132 (Bierman et al. 1992) for gene disruption, and pSET152 (Bierman et al. 1992) for gene complementation. The phenotype of the *S. lavendulae* FRI-5 strains was analysed after growth in liquid medium B, contained yeast extract 7.5 g, glycerol 7.5 g and NaCl 1.25 g per liter (Hashimoto et al. 1992). The media conditions and general *E. coli* and *Streptomyces* manipulations were as described previously (Kieser et al. 2000).

2.2.2 RNA isolation and gene expression analysis

Total RNA was prepared from mycelium grown in 70 ml of liquid medium B as described above by using an RNeasy Mini kit (Qiagen), and treated with DNase I (Takara Bio). IM-2 was

added to a final concentration of 100 nM at 5 h of cultivation, and cultivation was continued. The cDNA was synthesized using a GoScript^{TM} Reverse Transcription System (Promega KK) and Random Primers (Invitrogen) according to the manufacturer`s instructions. The cDNAs were amplified from the transcripts of the *far* regulatory genes using the previously described primers (Kitani et al. 2010). The primers used for the detection of the *hrdB* transcript were hrdB-Fw and hrdB-Rev, as listed in Table 2.1. The PCR amplification was performed by using GoTaq Green Master Mix (Promega KK) under the following conditions: 98ºC for 2 min, followed by discrete cycles of 98ºC for 30 s, 60ºC for 30 s, and 72ºC for 1 min. The absence of DNA contamination was confirmed by RT-PCR without reverse transcriptase.

Table 2.1 Oligonuclotides used in this study

Primer	**Sequence (5'-3')***
For genetic complementation of the *farR3*/*farR4* double disruptant	
farR3-Fw	GCTCTAGACAGCGCTCGATCCTGATGAGCA
farR3-Rev	GCTCTAGAGGTCAGGTCGGTGAGTTCCAGC
farR4-Fw	GCTCTAGACGTCTGCCTACCGAAAGTGCAG
farR4-Rev	GCTCTAGAGAGAAGCGCCAGGATCTGACGC
For RT-PCR analysis	
hrdB-Fw	TTCGAGGCTGACCAGATTCCT
hrdB-Rev	TCGCCCTCGTCCAGGTCCTTCTT

Restriction sites are underlined

2.2.3 Construction of the *farR3*/*farR4* double disruptant

The primers used for the construction of *S. lavendulae* FRI-5 mutant strains are listed in Table 2.1. The pMW102 (Kitani et al. 2008), including a 5.9-kb *Eco*RI fragment of the *farR4* downstream region, was cleaved by *Kpn*I and treated with T4 DNA polymerase to yield blunt

ends, and then a 3.0-kb (blunt-ended) *Nco*I fragment of the *farR4* upstream region obtained from the pMW101 (Kitani et al. 2001) was inserted into the *Eco*RV site of pBluescript II SK, resulting in pLT124. A 6.6-kb *Hin*dIII-*Nde*I fragment composed of regions upstream of *farR4* and downstream of *farR3*, recovered from pLT124, was blunt-ended and inserted into the *Eco*RV site of pKC1132 to generate pLT125 for *farR3*/*farR4* double disruption. *E. coli* ET12567 (pUZ8002) harbouring pLT125 was conjugated with *S. lavendulae* FRI-5 according to Kitani *et al*. (2000), and the wild-type gene was replaced with the disrupted allele [Δ*farR3*Δ*farR4* (ΔΔ)] by homologous recombination. The genotype of candidates for disruption of the two genes was confirmed by PCR analysis with the primer pair farR3-Rev/farR4-Fw under the following conditions 98ºC for 2 min, followed by 30 cycles of 98ºC for 30 s, 55ºC for 3 mins, and 72ºC for 1 min.

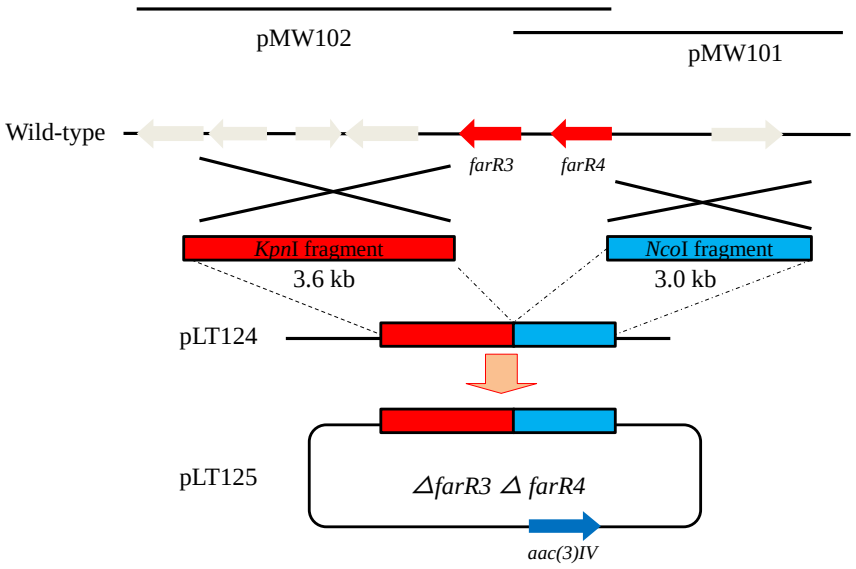


Figure 2.1 Schematic representation of the strategy for disruption of *farR3* and *farR4*. The red arrows represent the intact *farR3* and *farR4* gene. The red and blue bar represent *Kpn*I and *Nco*I fragments.

2.2.4 Genetic complementation of the *farR3*/*farR4* double disruptant

For complementation with both *farR3* and *farR4* genes, a 2.6-kb fragment containing the entire *farR3* and *farR4* genes with the 408-bp upstream region of *farR4* was PCR-amplified by the primer pair farR4-Fw/farR3-Rev, and then inserted into the *Eco*RV site of pBluescript II SK. The resulting plasmid was digested with *Xba*I, and then was cloned into the *Xba*I site of pSET152, resulting in pLT126. For complementation with either the *farR3* gene or *farR4* gene, the DNA fragments including the *farR3* gene or *farR4* gene with the 410-bp upstream regions of *farR3* and 408-bp upstream region of *farR4* were amplified by the primer pairs farR3-Fw/farR3-Rev or farR4-Fw/farR4-Rev, respectively, and then inserted into the *Eco*RV site of pBluescript II SK. The resulting plasmids were digested with *Xba*I, and were then cloned into the *Xba*I site of pSET152, a non-replicative vector, resulting in pLT127 or pLT128, respectively. The constructed plasmids were introduced into the *farR3*/*farR4* double disruptant by intergeneric conjugation and integration, mediated by intergeneric transfer of plasmids from *E. coli* to *Streptomyces* which integrate into the chromosome site-specifically at the ϕC31 attachment sites (Bierman et al. 1992). Integration of the plasmid was confirmed by apramycin resistance and PCR analysis using primers listed in Table 2.1 under the following conditions: 98ºC for 2 min, followed by discrete cycles of 98ºC for 30 s, 60ºC for 30 s, and 72ºC for 1 min.

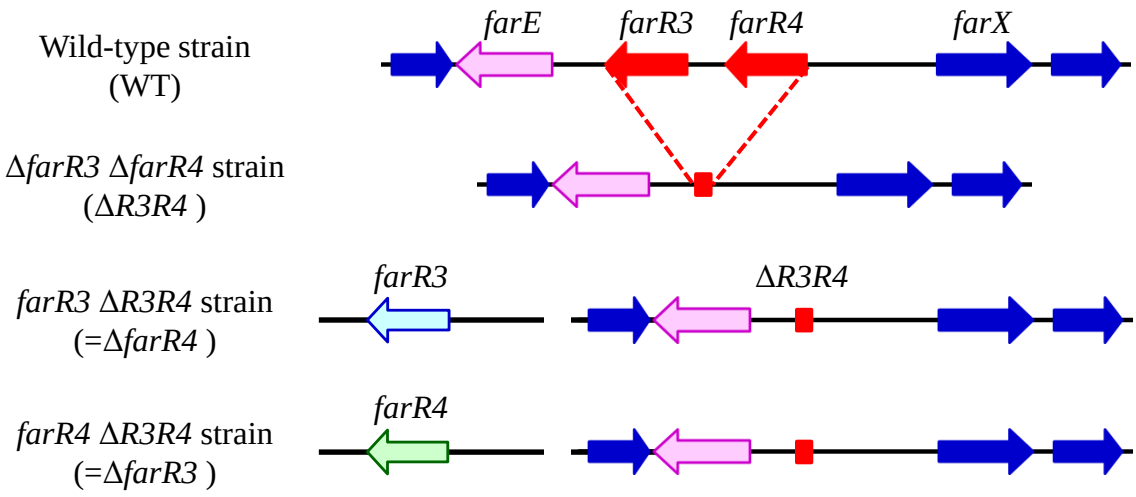


Figure 2.2 Schematic representation of the strategy for the simultaneous disruptions of the *farR3* and *farR4* genes

2.2.5 Analysis of blue pigment and other secondary metabolites

Pre-culture (2.5 ml) was inoculated into 70 ml of liquid medium B for analysis of blue pigment. Culture supernatants were collected periodically and filtrated through 0.2-μm-pore size filters, and the absorbance at 590 nm was measured for the production of blue pigment. The production of D-cycloserine and nucleoside antibiotics was measured as described by Kitani et al. (2001).

2.2.6 Isolation of the blue pigment

The culture supernatant (70 ml) after 8 h of cultivation with the exogenous addition of synthetic IM-2-C_5 (Hashimoto et al. 1992) at 5 h of cultivation was collected, and centrifuged (30,000 x *g*, 30 min) at 4^{o}C to recover the precipitated blue pigment. The pigment was washed twice with water and methanol and dried *in vacuo*. After dissolving in DMSO, the solution was filtered, and 5 volumes of water were added. By centrifugation at 30,000 x *g* for 30 min, the precipitated blue pigment was collected, washed three times with water and twice with methanol,

dried *in vacuo*, and dissolved in DMSO. The molecular mass of the blue pigment was analysed with a JEOL JMS-700 spectrometer by electron impact-mass spectrometry (EI-MS).

2.2.7 Analysis of IM-2 production

The culture supernatant (60 ml), which was prepared from the same preculture for the analysis of blue pigment was collected at the indicated times and adjusted to pH 3.0 with HCl. The supernatant was extracted three times with an equal volume of ethyl acetate. The ethyl-acetate extract was evaporated and dissolved in 3.5 ml of methanol as the sample for IM-2 assay. IM-2 activity was assayed by measuring the IM-2-dependent production of blue pigment (Yanagimoto and Enatsu 1983). One unit of IM-2 activity was defined as the minimum amount required for the induction of blue pigment production (Sato et al. 1989) and corresponded to 0.6 ng IM-2-C_5 ml^{-1} (2.97 nM).

2. 3 Results

2.3.1 Features of two putative regulatory genes, *farR3* and *farR4*

The putative regulatory genes *farR3* and *farR4* are in the centre of the 12.1-kb *far* regulatory island composed of seven regulatory genes (Kitani et al. 2008). FarR3 and FarR4 are significantly similar to the members of SARP-family regulatory proteins, which are characterized by an OmpR-type winged helix-turn-helix (HTH) DNA-binding domain at the N terminus containing two helical segments (α2 and α3) (Wietzorrek and Bibb 1997) and a bacterial transcriptional activator (BTAD) domain at the C terminus (Alderwick et al. 2006). SARP-family regulatory proteins generally act as activators for the production of secondary metabolites by directly activating transcription of the biosynthetic genes (Arias et al. 1999; Sheldon et al. 2002; Tanaka et al. 2007), and in most cases transcription of the regulatory gene is positively correlated

with that of the biosynthetic genes.

Previously, we found that *farR3* and *farR4* are transcribed in a growth-phase-dependent manner, and demonstrated an IM-2-dependent upregulation of transcription of both genes through the analysis of a *farX* disruptant lacking the intrinsic ability to synthesize IM-2 (Kitani et al. 2010). Under normal growth conditions of the wild-type strain with no IM-2 addition (Fig. 2.3b left, -IM-2), transcripts of both *farR3* and *farR4* were readily detected both at 6.5 h of cultivation (before IM-2 production at the early exponential phase) and at 8 h of cultivation, at which time points the blue pigment production had not yet been initiated, because the endogenous IM-2 concentration was insufficient for triggering secondary metabolism. The external addition of IM-2 at 5 h of cultivation (early-exponential growth phase) has been shown to induce blue pigment production from 7 h of cultivation (Kitani et al. 2001, 2010). With the external IM-2, the *farR3* transcription was clearly observed at 6.5 h but was significantly reduced at 8 h, whereas the *farR4* transcript became undetectable even at 6.5 h by the IM-2 addition (Fig. 2.3b right, +IM-2). To determine whether *farR3* and *farR4* are transcriptionally regulated by the IM-2 receptor FarA, we investigated the transcriptional profile in the *farA* disruptant (Fig. 2.3c). *farR3* was found to be transcribed at 5 h of cultivation in the *farA* disruptant, while the transcript was not detected in the wild-type strain. The transcriptional level of *farR3* decreased after 6.5 h and 8 h of cultivation regardless of whether IM-2 was added. On the other hand, *farR4* showed negligible transcription throughout the cultivation period in the *farA* disruptant. From these apparent influences of either IM-2 or the IM-2 receptor (FarA) on the transcription of *farR3* and *farR4*, we conclude that both *farR3* and *farR4* genes are under the transcriptional control of the IM-2/FarA regulatory system, which strongly suggests that these two genes might be involved in the regulation of secondary metabolism.

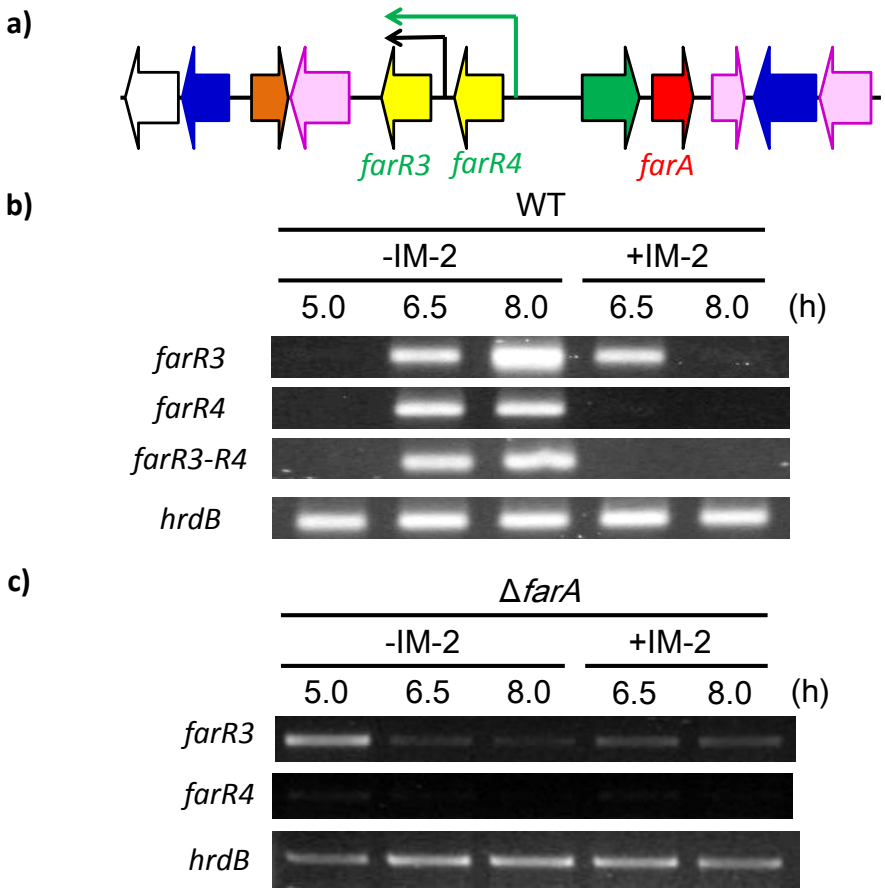


Figure 2.3 Transcriptional regulation on *farR3* and *farR4* by IM-2 and *farA*. (a) Organization of the *far* gene cluster in *S. lavendulae* FRI-5. The genes are indicated by arrows. The *farE* gene encodes a putative enzyme. The *farX* gene encodes an enzyme involved in IM-2 biosynthesis, and the *farA* gene encodes an IM-2-specific receptor. The bent arrows indicate transcriptional units of the *farR3* and *farR4* genes. (b and c) Transcriptional profiles of the *farR3* and *farR4* genes by semi-quantitative RT-PCR in the wild-type strain (b) and in the *farA* disruptant (c). Total RNAs were extracted from mycelia harvested at the indicated cultivation times without (-) or with (+) the exogenous addition of IM-2 at 5 h of cultivation. Indigoidine production was observed after incubation for 7 h with exogenous IM-2 addition. For PCR, 28 cycles of amplification were used for the transcripts of *farR3*, *farR4*, or the *farR4-farR3* operon, and 27 cycles for the *hrdB* transcript. The *hrdB*-like gene [*hrdB* encodes the major sigma factor in *Streptomyces coelicolor* A3(2)] was used as a control, because this gene is expressed fairly constantly throughout growth.

Because *farR4* and *farR3* run in the same transcriptional orientation with no plausible transcriptional terminator in the 3' region of *farR4* and the temporal expression profile of both genes are similar, as described above, the two genes may be organized in a bicistronic operon. RT-PCR analysis using RNA from the 6.5 h and 8 h mycelia with no addition of IM-2 revealed the expected transcripts containing the intergenic region of *farR4-farR3* (Fig. 2.3b). By contrast, the *farR4-farR3* transcript was not found in the RNA sample with the IM-2 addition, similar to the case of the *farR4* transcript. These findings led me to conclude that *farR4* is organized in a *farR4-farR3* bicistronic operon but *farR3* has an additional monocistronic mode of transcription by using its own promoter (Fig. 2.3a).

2.3.2. FarR3 positively regulates production of the blue pigment

To address the biological role of *farR3* and *farR4*, we first constructed a *farR3*/*farR4* double disruptant (Fig. 2.2) and compared its phenotypes to those of the wild-type *S. lavendulae* FRI-5. Growth in liquid culture and morphology on solid medium (formation of aerial mycelium and spores) of the double disruptant closely resembled those of the wild-type parent, indicating that FarR3 and FarR4 are not involved in primary metabolism or morphological development.

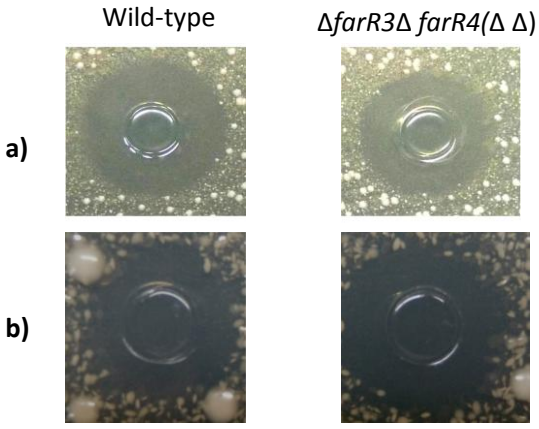


Figure 2.4 Bioassay for detection of D-cycloserine and nucleoside activity with *Bacillus subtilis*

as indicator strain. a) D-cycloserine and b) nucleoside antibiotics production in the wild-type and and *farR3*/*farR4* double disruptant.

Via activation of secondary metabolism by the addition of external IM-2, the wild-type strain began to produce blue pigment at 7 h of cultivation and showed the maximum production at 8 h (Fig. 2.5a, red-filled squares). In clear contrast, in the *farR3*/*farR4* double disruptant, the production of blue pigment was not detected at 7 h, but started at 8 h and reached a maximum at 9 h (37% of the wild-type maximum at 8 h) (Fig. 2.5a, green-filled squares). Complementation with an intact copy of the *farR3* and *farR4* genes containing the *farR4*-upstream region restored the phenotypes in the double disruptant to a level similar to that of the wild-type strain (Fig. 2.5a, blue-filled triangles). With respect to the production of D-cycloserine and nucleoside antibiotics, there was no difference between the wild-type strain and the *farR3*/*farR4* double disruptant (Fig. 2.4). These results indicated that the phenotypic change in the double disruptant was due to the loss of the *farR3*/*farR4* locus, implying that either *farR3* or *farR4*, or both, play a positive role in the regulation of IM-2-dependent blue-pigment biosynthesis. The blue pigment extracted as described in the Materials and Methods had a maximum UV absorption at 607 nm, which was consistent with that of indigoidine (Fig. 2.6a) (Kuhn et al. 1965; Starr et al. 1966). Furthermore, the EI-MS spectrometry showed a molecular ion peak at *m*/*z* 248 $[M]^+$ (Fig. 2.6b), corresponding to the molecular formula of indigoidine ($C_{10}H_8N_4O_4$), further confirming that the blue pigment produced in *S. lavendulae* FRI-5 is indigoidine.

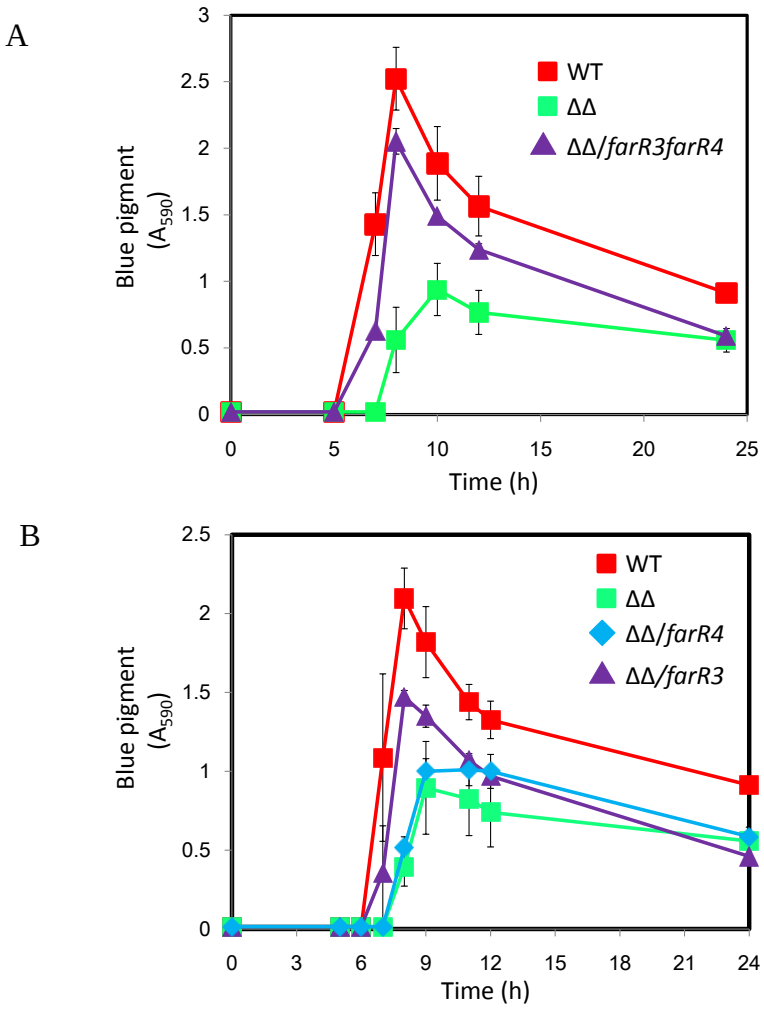
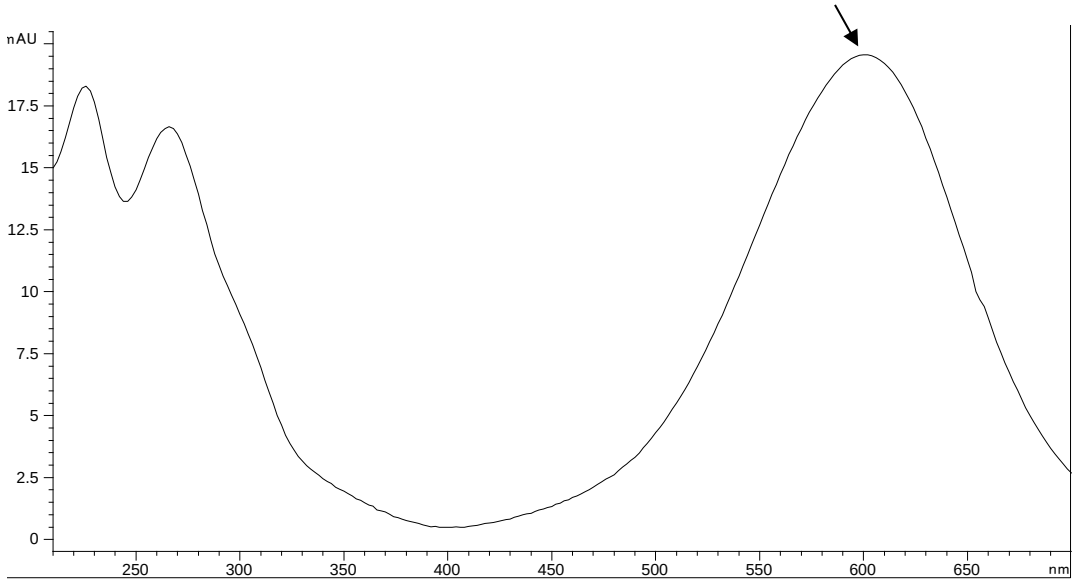


Figure 2.5 Inactivation of the *farR3* and *farR4* genes and genetic complementation of the double disruptant. WT, wild-type strain (red-filled squares); Δ*farR3* Δ*farR4* (ΔΔ)/-, *farR3*/*farR4* double disruptant (green-filled squares); ΔΔ/*farR4*, *farR4*-complemented ΔΔ strain (blue-filled diamonds); ΔΔ/*farR3*, *farR3*-complemented ΔΔ strain (purple-filled diamonds); ΔΔ/*farR3 farR4*, ΔΔ strain complemented with both *farR3* and *farR4* genes (blue-filled triangles). (a and b) Production profiles of indigoidine in the *farR3*/*farR4* double disruptant (a) and the double disruptant complemented with either the *farR4* or *farR3* gene (b) with the addition of exogenous IM-2 at 5 h of cultivation. Arrows indicate the timing of the IM-2 addition, and error bars represent standard deviations from triplicate experiments.

To determine which gene is responsible for the delayed and decreased production of indigoidine in the *farR3*/*farR4* double disruptant, we reintroduced plasmids that contained *farR3*

or *farR4*, respectively, with each upstream region into the double disruptant (Fig. 2.5b). Indigoidine production was found to be restored in a double disruptant complemented with *farR3* (ΔΔ/*farR3*) (Fig. 2.5b, purple-filled triangles), the genotype of which is equivalent to that of a conventional *farR4*-deletion mutant (Fig. 2.2). On the other hand, a double disruptant complemented with *farR4* (ΔΔ/*farR4*, a *farR3*-deletion mutant) still failed to produce indigoidine at 7 h and showed an indigoidine production profile similar to that of the *farR3*/*farR4* double disruptant as a parental strain (Fig. 2.5b, blue-filled diamonds). These results established that the phenotypic changes observed in the *farR3*/*farR4* double disruptant with respect to the indigoidine production came from the lack of *farR3*, not of *farR4*, and thus FarR3 can be concluded to function positively on the blue pigment (indigoidine) production temporally and quantitatively.

a)



b)

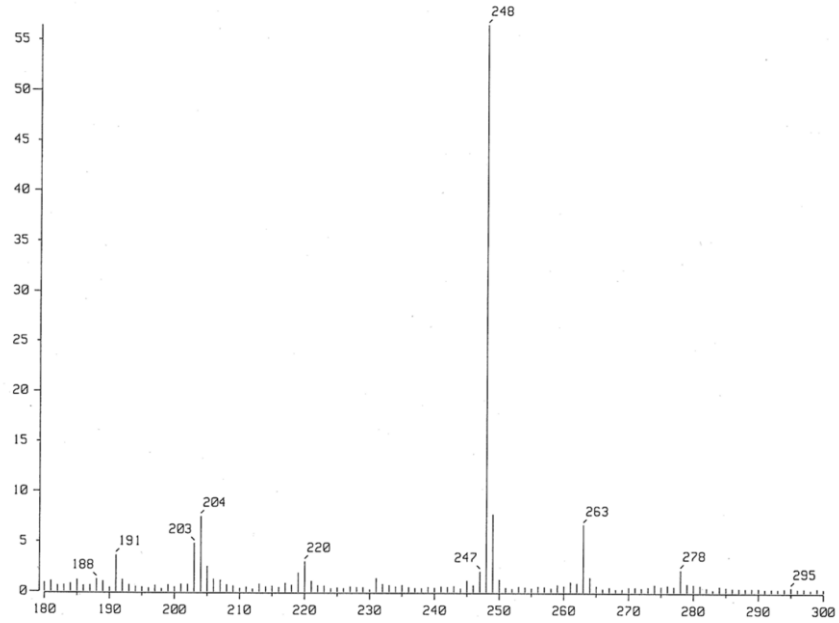


Figure 2.6 a) UV-Vis spectrum analysis of the blue pigment and b) EI-MS analysis of the blue pigment. The maximum UV absorption at 607 nm (black arrow) and peak m/z=248 were detected and was consistent with the maximum UV absorption and molecular weight of indigoidine ($C_{10}H_8N_4O_4$).

2.3.3 Indigoidine production in the *farR4*-deficient strain

As shown in Fig. 2.3b, *farR4* was transcribed before IM-2 production. In addition, when IM-2 was added, the ΔΔ/*farR3* (equivalent to Δ*farR4*) strain produced indigoidine at a level similar to the wild-type strain. These observations suggested that *farR4* might exert its function primarily before the perception of IM-2. To further explore this possibility, indigoidine production in the ΔΔ/*farR3* strain was investigated with no external IM-2. In the wild-type strain, indigoidine production started only at 10 h in response to the biosynthesis of endogenous IM-2, and declined in a growth-dependent manner (Fig. 2.7). However, the ΔΔ/*farR3* strain showed indigoidine production at 8 h and reached a level of production similar to that of the wild-type strain at 9 h, indicating that the lack of *farR4* caused 2-h earlier onset. The production of D-cycloserine and

nucleoside antibiotics both in the ΔΔ/*farR3* strain and in the *farR3*/*farR4* disruptant was identical to those of the wild-type strain. These results indicated that, unlike FarR3, FarR4 has a negative effect on the production onset of indigoidine.

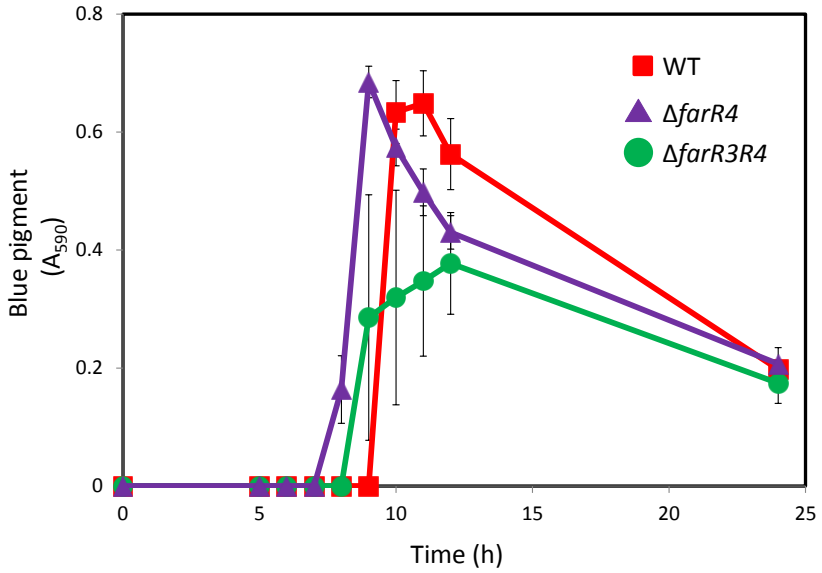


Figure 2.7 Production profiles of indigoidine in the *farR3*-complemented ΔΔ strain without the addition of exogenous IM-2. Red-filled squares, green-filled circles, and purple-filled triangles indicate the time courses of indigoidine production in the wild-type strain, ΔΔ strain, and *farR3*-complemented ΔΔ strain, respectively.

2.3.4 Transcriptional analysis of *far* regulatory genes in the *farR4*-deficient strain

The effect of each regulatory gene on the expression of other regulatory genes in the *far* region was monitored by semiquantitative RT-PCR analysis in the *farR3*/*farR4* double disruptant, in the ΔΔ/*farR3* strain, and in the ΔΔ/*farR4* strain, respectively (Fig. 2.8). Total RNA was isolated at 6.5 h of cultivation, at which time point the IM-2 concentration had not yet reached a threshold for triggering secondary metabolism. At the cultivation time, transcripts of *farR1*, *farR3*, *farR4*, and *farA* were detected in the wild-type strain, whereas the transcription levels of both *farR1* and *farR2* were significantly enhanced by gene deletion of *farR3* and *farR4*. The double

disruptant also showed increased transcription of *farA* compared to that of the wild-type strain. Interestingly, in addition to the transcripts of these three regulatory genes, the *farX* transcript was apparent in the double disruptant but was not present in the wild-type strain. The gene expression profile of the ΔΔ/*farR3* strain was identical to that in the double disruptant. In contrast, the transcription levels of these four genes in the ΔΔ/*farR4* strain were almost the same as those in the wild-type strain, although the *farR4* gene introduced by *in trans* complementation was expressed at a slightly higher level than that in the wild-type strain. These results indicated that transcriptional upregulation in the *farR3*/*farR4* double disruptant was due to the lack of *farR4*, suggesting that FarR4 negatively controls the expression of three regulatory genes (*farA*, *farR1*, and *farR2*) and an IM-2 biosynthetic gene (*farX*) which are pivotal factors at the top of the IM-2 signalling cascade.

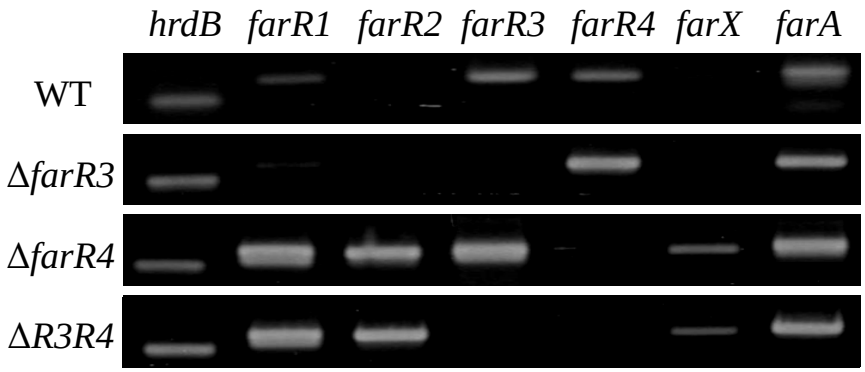


Figure 2.8 Gene expression analysis of *far* genes by semi-quantitative RT-PCR in the wild-type strain (WT), ΔΔ strain (ΔΔ), *farR4*-complemented ΔΔ strain (ΔΔ/*farR4*), and *farR3*-complemented ΔΔ strain (ΔΔ/*farR3*). Total RNAs were isolated from mycelia harvested at 6.5 h of cultivation with no IM-2 addition. In the PCR, 26 cycles of amplification were used for the *farX* transcript, 28 cycles for the transcripts of the other genes and 27 cycles for the *hrdB* transcript.

2.3.5 Negative regulation of IM-2 biosynthesis by FarR4

Because indigoidine production in the ΔΔ/*farR3* (equivalent to Δ*farR4*) strain started at an earlier cultivation time than that in the wild-type strain and lack of *farR4* induced the expression of *farX*, the IM-2 biosynthesis was investigated (Fig 2.9).

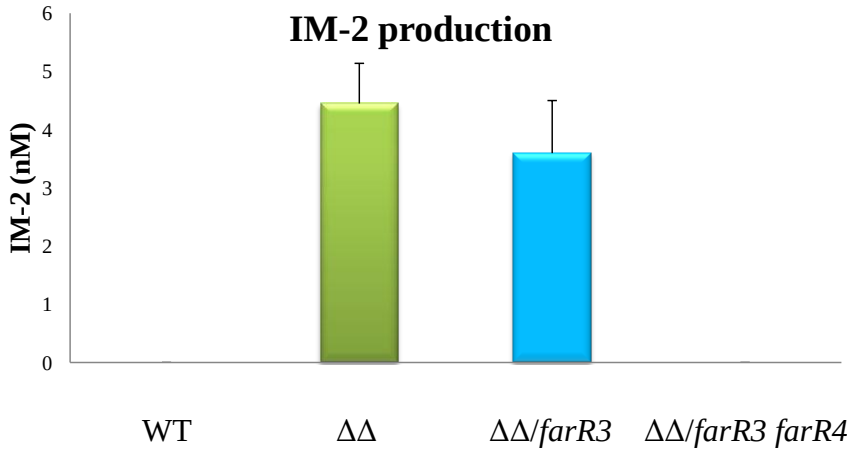


Figure 2.9 IM-2 production of the *S. lavendulae* FRI-5 wild-type strain and a *farR3/farR4* double disruptant.

After 7 h of cultivation, no IM-2 activity (detection limit 0.6 nM) was detected in the culture broth of the wild-type strain (WT) or in the *farR3*/*farR4*-complemented *farR3*/*farR4* double disruptant (ΔΔ/*farR3 farR4*). However, the ΔΔ/*farR3* strain and the double disruptant (ΔΔ) produced IM-2 at 3.6 nM and 4.5 nM in the culture broth, respectively. After 8 h of cultivation, IM-2 activity was also detected in the samples of all four strains tested. These results indicated that the lack of *farR4* elicited ectopic expression of FarX and earlier production of IM-2, which in turn resulted in earlier production of indigoidine. Thus, FarR4 should be concluded to act as a negative regulator of IM-2 production through the transcriptional control of the IM-2 biosynthetic gene, *farX*.

2.4 Discussion

The isolation and functional analysis of regulatory genes operating in the IM-2-FarA stimulon from *S. lavendulae* FRI-5 have helped unravel the γ-butyrolactone autoregulator signalling cascades in streptomycetes. We previously showed that *farR3* and *farR4*, encoding homologues of the SARP-family of antibiotic regulators, are present in the *far* regulatory island together with five other regulatory genes, and suggested that they are probable members of the IM-2 stimulon involved in the regulation of secondary metabolism (Kitani et al. 2008, 2010). In this study, I demonstrated that the expression of both FarR3 and FarR4 is under the control of the IM-2/FarA system, and verified that they have distinct contributions to the regulation of secondary metabolism: FarR3 is a positive regulator of indigoidine biosynthesis, and FarR4 plays a negative regulatory role in the IM-2 biosynthesis. However, it remains unclear whether FarR3 or FarR4 act as a direct transcriptional activator or repressor on the biosynthetic genes for indigoidine or IM-2, respectively.

The SARP-family regulators are known to be pathway-specific regulators that usually act as a master switch to initiate biosynthesis of individual secondary metabolites, and are regarded as the lowest-level regulators in the regulatory cascade. During the activation of secondary metabolism by IM-2, FarR3 positively controls not only the amount of indigoidine but also the onset of the indigoidine production (Fig. 2.5). Because lack of *farR3* did not show any effect on the expression of *farA* and other regulatory genes (Fig. 2.10), FarR3 can be concluded to be a typical pathway-specific regulator for indigoidine production at the lowest-level of the IM-2/FarA regulatory cascade.

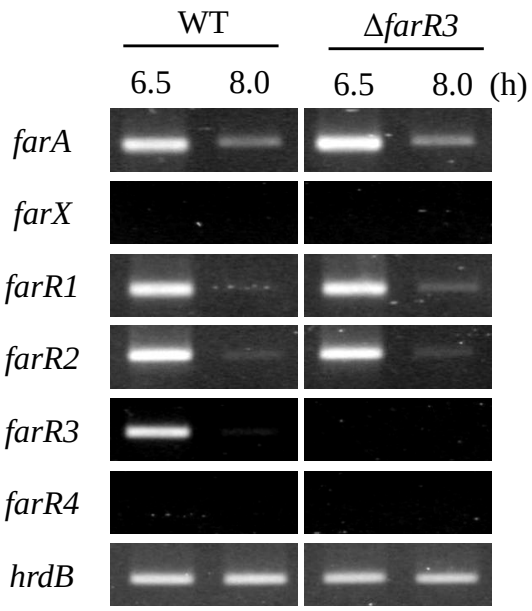


Figure 2.10 Gene expression analysis of *far* genes in the wild-type strain (WT) and the *farR4*-complemented ΔΔ strain (ΔΔ/*farR4*) with *hrdB* as positive control. Total RNAs were extracted from mycelia harvested at the indicated cultivation times with the exogenous addition of IM-2 at 5 h of cultivation

Unlike the function of FarR3, the function of FarR4 is to suppress biosynthesis of IM-2 by controlling transcription of the principal IM-2 biosynthetic gene, *farX*. To date, the molecular mechanisms underlying the control of γ-butyrolactone biosynthesis remain poorly understood, although pseudo γ-butyrolactone receptors have recently been reported to be involved in the control of γ-butyrolactone biosynthesis (Wang et al. 2011). This is the first report to note that a SARP-family regulator modulates biosynthesis of a signalling molecule such as γ-butyrolactone, which is situated at the highest level in the regulatory cascade of *Streptomyces* secondary metabolism, indicating an autoregulatory circuit for γ-butyrolactone biosynthesis.

The genes encoding SARP-family regulators are frequently located inside or adjacent to their own biosynthetic gene clusters, and their products mediate the positive transcriptional effect

on the biosynthetic genes by binding to the promoter region of the target genes. We have already found that a putative indigoidine biosynthetic gene [significant identity (98%) and similarity (99%) with BpsA from *Streptomyces lavendulae* ATCC11924, which is involved in the biosynthesis of indigoidine (Takahashi et al. 2007)] is present in the left-hand region approximately 24 kb away from the *farR3*/*farR4* locus (our unpublished data). During analysis of a *farX* promoter region, I identified the transcriptional start point of *farX* as G, situated at 116 nt upstream from the translational start codon of FarX (data not shown). However, the plausible binding site of the SARP-family regulators, which are direct heptameric sequences (TCGAGXX) spaced by 4 or 15 nt (Tanaka et al. 2007), is not found either in the upstream regions of the putative indigoidine biosynthetic gene nor in the promoter region of *farX*, suggesting that FarR3 and FarR4 may operate via another regulator to regulate the biosynthesis of indigoidine and IM-2, respectively. However, I cannot rule out the possibility that FarR3/FarR4 might recognize a region distinct from the canonical binding sites of the SARP-family regulator, because there are some exceptions from this characteristic (Yu et al. 2012).

IM-2 production is prominently observed in the late exponential phase and declined gradually in a growth-dependent manner (Yanagimoto et al. 1988). Before IM-2 production, the *farR3* and *farR4* genes were transcribed as a bicistronic operon. The exogenous addition of IM-2 abolished the *farR4-farR3* transcript, suggesting that the promoter activity of *farR4* was downregulated by IM-2 when the IM-2 concentration reached a threshold level to trigger the production of secondary metabolites at the stationary phase. On the other hand, *farR3* was still transcribed even when the bicistronic operon disappeared in the presence of IM-2. Taken together with the precocious expression of *farR3* in the *farA* disruptant (Fig. 2.3c), these results suggest that the transcriptions of *farR3* and *farR4* are operated by several layers of regulation of the IM-2/FarA system (see below), and *farR3* expression is temporally governed by two different

promoter regions in response to IM-2 and FarA. Our previous studies have shown two different roles of FarA on transcriptional regulation of the target genes (Kitani et al. 2001, 2008). First, FarA represses the transcription of the target genes via binding to FAREs, and the binding of IM-2 to FarA allows these genes to be unregulated via the release of the IM-2-FarA complex from FAREs. Secondly, the IM-2-FarA complex itself has a DNA-binding activity for controlling expression of the target genes, although the *in vitro* function of the IM-2-FarA complex remains to be clarified. With respect to the indigoidine production, FarA is the negative regulator suppressing the biosynthesis of indigoidine before the IM-2 production, which could be explained by the former regulatory mode of FarA.

The *far* regulatory island showed a variety of putative gene functions similar to that found in *Streptomyces aureofaciens* (Fig. 2.11b) (Novakova et al. 2010). Interestingly, Aur1PR3, which presumably shares a common evolutionary origin with FarR4, controls the production of auricin quantitatively with a positive function, whereas Aur1PR2, the FarR3 homologue lacking several amino acids in the DNA-binding domain, has no function in the regulation of auricin production (Novakova et al. 2011). Despite the highly conserved gene arrangement and high similarity, regulatory proteins that belong to the same family exert different regulatory activities, suggesting that each of the *Streptomyces* species has its own complex regulatory mechanism for secondary metabolite production. Based on my current results, indigoidine biosynthesis is tightly regulated by two SARP-family proteins acting at different levels of the IM-2/FarA regulatory cascade. At early stages of growth before the IM-2 production, *farA* is autorepressed by FarA formed from basal-level transcription, forming an autoregulatory circuit (Kitani et al. 1999). At that time, FarA negatively regulates transcription of *farR3* and therefore indigoidine production is not initiated. The expression of *farR3* increases gradually in a growth-dependent manner by an unknown mechanism, and is notably relieved in response to IM-2 production at the late exponential phase

to initiate indigoidine biosynthesis.

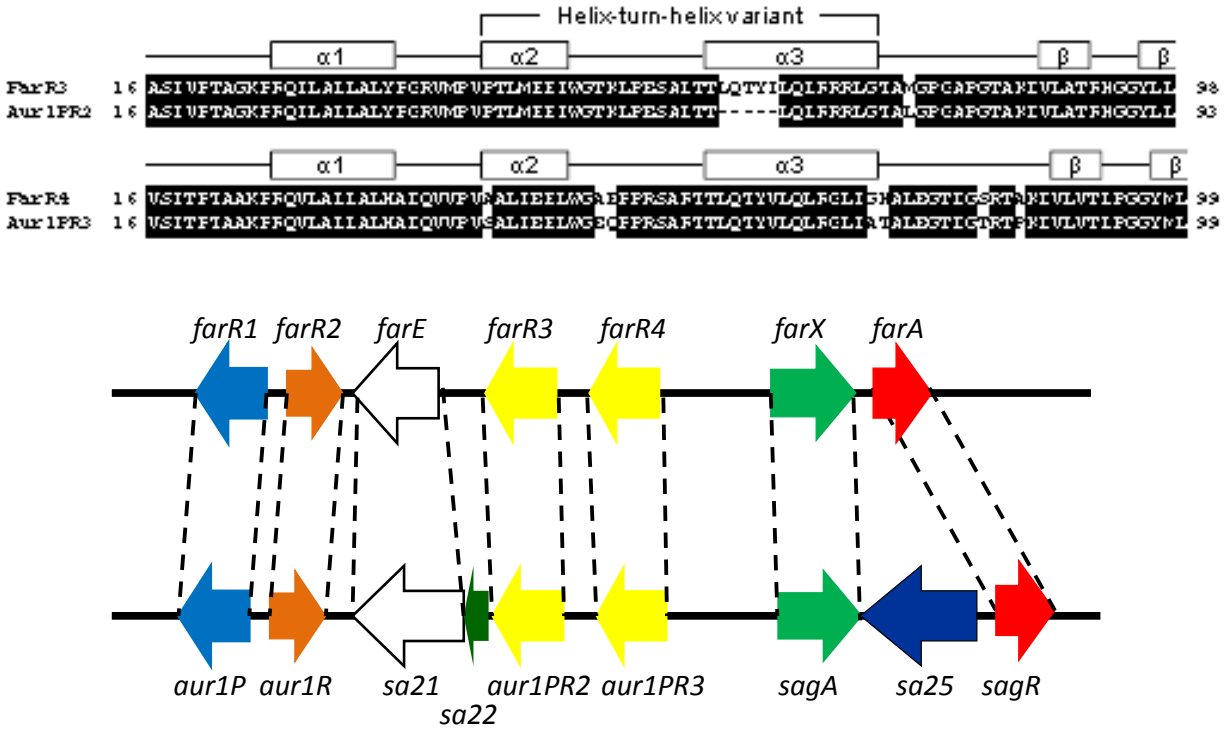


Figure 2.11 (**a**) Sequence alignment of the N-terminal regions of FarR3 with Aur1PR2 (upper panel) and of FarR4 with Aur1PR3 (lowerpanel). The numbers indicate the amino acid positions within each sequence. Identical residues are highlighted in black. The predicted secondary structure elements for the SARP family members are shown above their sequences. (**b)** Comparison of the *far* gene cluster in *S. lavendulae* FRI-5 with the *S. aureofaciens* CCM 3239 chromosomal regionencoding regulatory proteins for auricin production.

In contrast, transcription of *farR4* seems to be positively regulated by the basal level of FarA at the early exponential phase, because the transcript disappeared in the *farA* disruptant (Fig. 2.3c). At the mid-exponential phase, the transcription of *farR4* increases gradually similar to the case of *farR3*, whereas another regulation should operate to override the repressing activity of FarR4 on *farX* to ensure sufficient production of IM-2 by FarX, which indicates an exquisite regulation in the early process of IM-2 production. Thus, FarR4 is an important determinant for controlling the initiation time of IM-2 production. Taken together with the model proposed

previously for a negative autoregulatory circuit for IM-2 production at the stationary phase (Kitani et al. 2010), my findings suggest that *S. lavendulae* FRI-5 has a fine-tuning system to control γ-butyrolactone biosynthesis. Further understanding how the *farR3*/*farR4* genes are controlled by the IM-2/FarA system and how they regulate indigoidine production or IM-2 biosynthesis will shed new light on the regulation of γ-butyrolactone biosynthesis as well as the regulatory networks for secondary metabolism in streptomycetes.

2.5 Summary

The *Streptomyces* antibiotic regulatory protein (SARP) family regulators have been shown to control the production of secondary metabolites in many *Streptomyces* species as the most downstream regulators in the regulatory cascade. *Streptomyces lavendulae* FRI-5 produces a blue pigment (indigoidine) together with two types of antibiotics: D-cycloserine and the nucleoside antibiotics. The production of these secondary metabolites is governed by a signaling system consisting of a γ-butyrolactone, IM-2 [(2*R*,3*R*,1'*R*)-2-1'-hydroxybutyl-3-hydroxymethyl-γ-butanolide], and its cognate receptor, FarA.

Here, I characterized two regulatory genes of the SARP family, *farR3* and *farR4*, which are tandemly located in the proximal region of *farA*. *farR3* is transcribed both as a monocistronic RNA and as a bicistronic *farR4-farR3* mRNA, and the expression profile is tightly controlled by the IM-2/FarA system. Loss of *farR3* delayed and decreased the production of indigoidine without any changes in the transcriptional profile of other *far* regulatory genes, indicating that FarR3 positively controls the biosynthesis of indigoidine, and is positioned in the downstream region of the IM-2/FarA signalling system. Meanwhile, loss of *farR4* induced the early production of IM-2 by increasing transcription of an IM-2 biosynthetic gene, *farX*, indicating that FarR4 negatively controls the biosynthesis of IM-2. Thus, our results suggested differential

contributions of the SARP-family regulators to the regulation of secondary metabolism in *S. lavendulae* FRI-5. This is the first report to show that an SARP-family regulator is involved in the biosynthesis of a signaling molecule functioning at the most upstream region of the regulatory cascade for *Streptomyces* secondary metabolism.

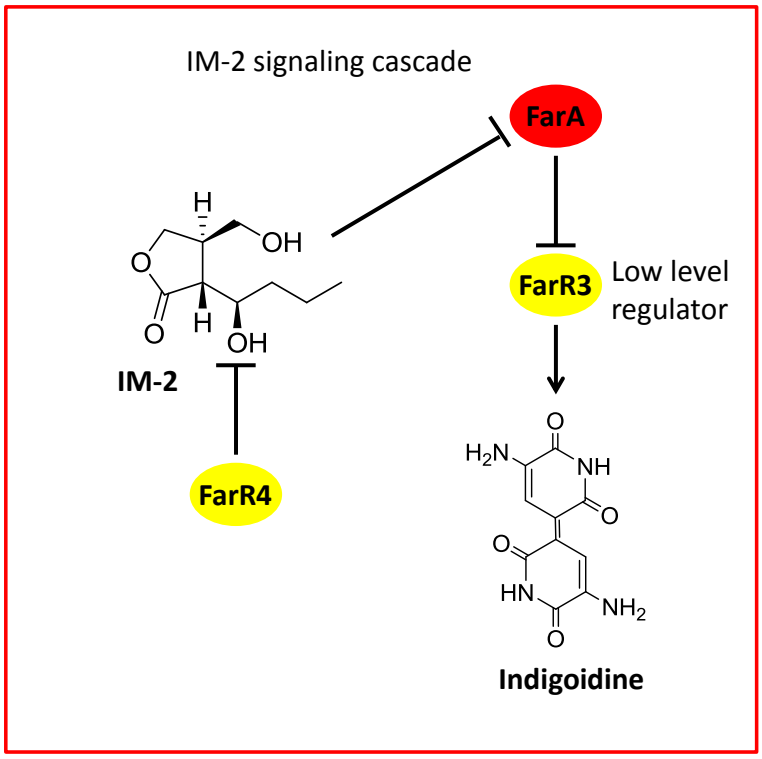


Figure 2.12 A simplified model of differential contributions by FarR3 and FarR4 to blue-pigment indigoidine biosynthesis.

Chapter 3

Regulatory roles of a pseudo-γ-butyrolactone receptor on secondary metabolism of *Streptomyces lavendulae* FRI-5

3.1 Introduction

The Gram-positive, soil-inhabiting filamentous bacterial genus streptomyces has been well known for decades because of their complex life cycle and ability to synthesize a wide range of bioactive secondary metabolites possessing antibiotic or other useful pharmacologic activities, including anticancer, antitumor, and immunosuppressive activities. The production of these bioactive secondary metabolites is usually tightly regulated in a hierarchical manner at several layers. This involves higher-level regulators regarded as global regulators mediating stimuli from the environment, thus controlling the activity of the pathway-specific regulators that directly control the activation of biosynthetic genes for secondary metabolites (Bibb 2005; Liras et al. 2008).

In streptomyces, the γ-butyrolactone (GBL) system (Folcher et al. 2001, Bibb 2005), typically consisting of a GBL molecule and a cognate receptor protein, is of great interest by virtue of their significant roles in regulating the production of bioactive secondary metabolites. Despite their importance in regulating secondary metabolism, many aspects of the GBLsignal are still poorly understood. The best characterized component of the system is the A-factor−mediated signaling system in *Streptomyces griseus* (Horinouchi 2002). Before the A-factor is produced, the cognate receptor ArpA binds to the promoter region of a pleiotropic regulatory gene, *adpA*, and represses its transcription. When A-factor is produced and reaches a threshold concentration, ArpA dissociates from the promoter region of *adpA* by binding with A-factor, leading to the

transcription of *adpA*. AdpA then activates the transcription of many genes that are required for morphological differentiation and secondary metabolism (i.e., streptomycin production), forming an AdpA regulon.

To date, many genes encoding autoregulator receptors have been reported to control the production of secondary metabolites (Nakano et al. 1998, 2000; Kitani et al. 2001; Takano et al. 2001). In most cases, an autoregulator receptor gene, often accompanied by pathway-specific regulatory genes and genes encoding a homologue of the autoregulator receptor, is found within a biosynthetic gene cluster of secondary metabolite, and they operate at several regulation layers for the biosynthesis of the cognate secondary metabolite through the transcriptional regulation

Genome information on the sequenced streptomyces revealed the existence of many GBL receptor homologues, and gives rise to the question of whether they are also involved in the GBL-mediated signaling cascade (Nishida et al. 2007). Some studies revealed that GBL receptor homologues participate in the regulation mechanism, mainly acting as a repressor of the biosynthesis of secondary metabolites (Novakova et al. 2010; Bunet et al. 2011). Interestingly, these GBL receptor homologues do not exhibit any gamma-butyrolactone binding ability and are thereby designated "pseudo" GBL receptors (Bunet et al. 2011). Apart from not having the ability to bind the GBL, pseudo-GBL receptors are also characterized by high p*I* values, in contrast to the low-acidic p*I* values of true GBL receptors. It has been reported that pseudo-GBL receptors coordinate antibiotic biosynthesis by binding and responding to antibiotic signals, suggesting that antibiotics can also act as intracellular signals to induce downstream processes (Xu et al. 2010). The same study also reported the possible correlation between pseudo-GBL receptors and silent antibiotic clusters, as the inactivation of two pseudo-GBL receptors led to the production of two cryptic metabolites. A more recent study reported a novel role for pseudo-GBL receptors: involvement in the control of GBL biosynthesis by directly repressing the transcription of genes

that encode the key enzymes for GBL biosynthesis (Wang et al. 2011). Due to the wide range of actions of pseudo-GBL receptors, a study on their roles in the GBL-mediated signaling cascade will be of great interest and will provide better knowledge of the GBL signaling cascade while also serving as an important tool with which to isolate novel but cryptic natural products.

Streptomyces lavendulae FRI 5, which produces blue pigment indigoidine, nucleoside antibiotics, and the anti-tuberculosis drug D-cycloserine, employs a GBL system to control the production of the aforementioned secondary metabolites. The GBL system in *S. lavendulae* FRI 5 consists of GBL molecule IM-2 and cognate receptor FarA (Hashimoto et al. 1992; Waki et al. 1997). Unlike other GBL molecules that play positive roles in the regulation of secondary metabolite production, IM-2 exerts both positive and negative effects on the regulation of secondary metabolism; namely, it switches on the production of indigoidine and nucleoside antibiotics and switches off the production of D-cycloserine (Hashimoto et al. 1992). IM-2 production preceded secondary metabolite production, initiated before the mid-exponential phase, reached the threshold concentration at the mid-exponential phase, and triggered secondary metabolite production at the late exponential phase (Yanagimoto et al. 1998). *farA* and a biosynthetic gene for IM-2 biosynthesis, *farX*, are located in the same locus in the 12-kb *far*-regulatory region. In addition to the two regulatory genes, four more regulatory genes (*farR1, farR2, farR3,* and *farR4*) are present in the *farA* flanking region (Kitani et al. 2008). In chapter 2, I reported the differential contributions of FarR3 and FarR4, belonging to the *Streptomyces* Antibiotic Regulatory Protein (SARP), to the IM-2 signaling cascade; FarR3 positively controls indigoidine production and FarR4 negatively controls IM-2 biosynthesis (Kurniawan et al. 2014)

In silico and phylogenetic analyses indicated that the product of *farR2* is a pseudo-GBL receptor due to its high degree of similarity with FarA and its high p*I* value of 9.7. We previously found that *farR1* and *farR2* are the direct transcriptional targets of FarA through the binding of

FarA to FAREs (FARE2 and FARE3, in the promoter region of *farR1* and *farR2*) (Kitani et al. 2008). The binding was relieved by IM-2, as demonstrated by gel retardation assays in which the addition of synthetic IM-2 to a cocktail mixture of FarA and a labeled probe dissociated the IM-2-FarA complex. Whereas in the wild-type strain *farR2* was transcribed from the mid-exponential phase, the transcriptional analysis in a mutant strain lacking the ability to synthesize IM-2 (obtained by deletion of *farX*) showed that the transcription of *farR2* was completely abolished (Kitani et al. 2010). These findings promoted us to investigate the function of FarR2 in *S. lavendulae* FRI-5, which might form a more complicated γ-butyrolactone regulatory system for secondary metabolism compared to that of other *Streptomyces* species.

In the present study, we characterized that FarR2 belongs to the pseudoreceptor regulator in the IM-2/FarA system, and demonstrated that FarR2 positively controls the initiation timing of indigoidine production in response to the presence of IM-2 and is involved in the transcriptional repression of the *far* regulatory genes at the late stage of secondary metabolism, implying the functional diversity of the pseudoreceptor regulator in streptomycetes.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids, and growth conditions

S. lavendulae FRI-5 (MAFF10-06015; National Food Research Institute, Tsukuba, Japan) was grown on ISP medium 2 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for spore formation. *Escherichia coli* DH5*α* was used for general DNA manipulation (Sambrook et al. 2001), and the DNA methylation-deficient *E. coli* strain ET12567 containing the RP4 derivative pUZ8002 (Paget et al. 1999) was used for *E. coli/Streptomyces* conjugation. The plasmids used were pBluescript II SK and pUC19 for general cloning, pKC1132 (Bierman et al. 1992) for gene

disruption, pENTR (Invitrogen, CA, USA) and pLT101 (Pulsawat et al. 2009) for gene complementation. The phenotype of the *S. lavendulae* FRI-5 strains was analyzed after growth in liquid medium B (Hashimoto et al. 1992). The media conditions and general *E. coli* and *Streptomyces* manipulations were as described previously (Kieser et al. 2000). The primers used in this study are listed in Table 3.1, except for the primers described in the previous reports.

Table 3.1. Oligonucleotides used in this study

Primer	**Sequence (5'-3')***
For transcriptional analysis of truncated *farR2*	
tfarR2-Fw	TGGCGGCCACCCTCGGCTTC
For construction of *farR2* disruptant and *farR2*-complemented strain	
farR2-up-Fw	CCCAAGCTTTTCAACAGCGCTGGATTCAGGA
farR2-up-Re	ACATGCATGCACGGCGCGTTCTTGTTCAT
farR2-down-Fw	ACATGCATGCACTCAACCACCGCAACGTG
farR2-down-Re	GCTCTAGATGGACGTCCACACCTACGA
farR2-comp-Fw	CACCTGATACAGGAGCTATGCGTGA
farR2-comp-Re	TCAGCTCCCCCTTCCGGCCGTGTCC
dfarR2Fw	AATCGCCGTTGATCAGGCCACCAA
dfarR2Re	ACGGCAGAGAACGTGTCAGCT
For overexpression of recombinant FarR2 and FarA proteins	
rFarR2-Fw	GCATATGAAACAGGAACGCGCCGTCCGCAC
rFarR2-Re	CAAGCTTGCTCCCCCTTCCGGCCGTGTC
rFarA-Fw	GCATATGGCTGAACAGGTCCGAGCCATC
rFarA-Re	CAAGCTTGTCTTCCTCGTCCGCCTGCTCC

For gel shift assay

F1-Re	GGGATTCTGGCGCGTGCGGATGGCTC
F11Fw	GGAATTCGATACGAACGGGACGGACGG
F12-Fw	GATACGCGATAGACGGACGGTTTGCAG
F13-Fw	GGAATTCAGGGCTTCCGACGCTCCGAAC
F2-Re	GGAATTCGGTGGTGTGAGGTTCCAGTAC
F21-Fw	GGGGATCCCTGATCAACGGCGATTCGTGC
F22-Fw	GGGATCCTTTGACAAACCGACGAAGCG
F23-Fw	TTTGACGCGATAACGAAGCGGTTTG
F24-Fw	GGGATCCGTTGACGTTGGCTCAACTGAG
F3-Re	GGGGATCCTGACTCGAGCAGTGCTTGACG
F31-Fw	GGAATTCGTAGATCAAATCAGGCCACAGC
F32-Fw	GGAATTCCTCTTCCAATACCAACGGG
F33-Fw	GGAATTCCTCTTCCAATTGCAACGGG
F34-Fw	GGAATTCGAAACTGATACAGGAGCTA
F4-Re	GGGGATCCCCGTTCTCCCGCACGGTCAG
F41-Fw	GGAATTCCGCCCGATGATCGTCAGACC
F42-Fw	GGGATTCTATTGACAAACCGGCGCAGC
F43-Fw	TATTGACGCGATAGCGCAGCTGTTTTTCC
F44-Re	CCAAACCGGCTGTACCGTTTTTTGCTC
F45-Re	CCGGGAATGCTGTACCGTTTTTTGCTCC
F46-Fw	CCTCGTTCCAGCGAACTTCGAGGGTTC
F6-Re	GGGATCCGGGAAGCAGGAGTCGTGCAG
F61-Fw	GGAATTCGAAAAAACCGGTTGGTATAT
F62-Fw	GAAAGGGAATGTTGGTATATATTTTTCTG
F63-Fw	GGAATTCCTGAGCAGTCACACCTGTTAC

For 5'-RACE

farX-GSP1	ATCCAGCCGCTGCCAGTCGGTGAG
farX-GSP2	CGTGGACGTGCGGTGTACCAGTTC

*Restriction sites are underlined

3.2.2 Construction of the *farR2* disruptant

A 2.0 kb *farR2*-upstream fragment was amplified by the primer pair farR2-up-Fw/farR2-up-Re, and digested with *Hin*dIII and *Sph*I. Similarly, a 2.2 kb *farR2*-downstream fragment was amplified by the primer pair farR2-down-Fw/farR2-down-Re, and digested with *Sph*I and *Xba*I. The two resulting fragments were cloned together into the *Hin*dIII and *Xba*I sites of pUC19, and were recovered as a 4.2 kb *Hin*dIII/*Xba*I fragment. The fidelity of the amplified region was confirmed by DNA sequencing. The 4.2 kb fragment was inserted into the *Hin*dIII and *Xba*I sites of pKC1132 to generate pLT131 for *farR2* disruption. *E. coli* ET12567 (pUZ8002) harboring pLT131 was conjugated with *S. lavendulae* FRI-5, and the wild-type gene was replaced with the disrupted allele (Δ*farR2*) by homologous recombination. The genotype of the Δ*farR2* candidates was confirmed by PCR analysis with the primer pair dfarR2Fw/ dfarR2Re under the following conditions: a single round of 98^{o}C for 2 min and 30 cycles of 98^{o}C for 30 s, 55^{o}C for 30 s, and 72^{o}C for 1 min. The *S. lavendulae* FRI-5 *farR2* disruptant was abbreviated Δ*farR2*.

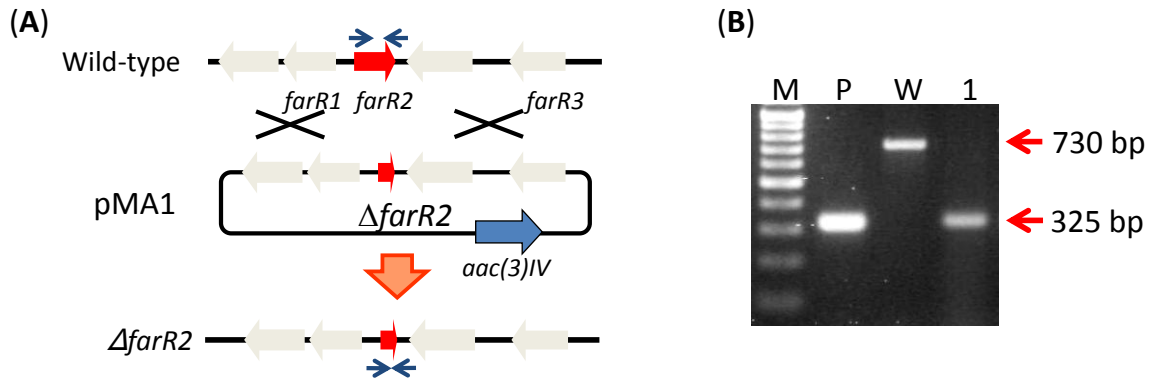


Figure 3.1 Schematic representation of the strategy for disruption of *farR2*. The red arrows (long and short) represent the intact *farR2* gene and the disrupted *farR2* gene (*ΔfarR2*), respectively. Outward and inward blue arrows represent the primer pair used to analyze the genotype of *ΔfarR2*candidates. PCR analysis of chromosomal DNA from the wild-type strain (W), the *ΔfarR2* strain (lane 1). M= Marker; P= Control.

3.2.3 Complementation of the *farR2* disruptant

A Gateway Reading Frame Cassette C.1 (Invitrogen) was cloned into the blunt-ended *Xba*I site of pLT101 to yield pLT114 as a destination vector. The entire *farR2* gene with its 30-bp upstream region including the putative ribosome-binding site was amplified by using the primer pair farR2-comp-Fw/farR2-comp-Fw, and then cloned into a pENTR vector to generate an entry clone. The entry clone was used with pLT114 in an LR reaction (LR Clonase Enzyme Mix; Invitrogen), resulting in pLT132. The plasmid pLT132 was introduced into the Δ*farR2* strain by intergeneric conjugation and integration. Integration of the plasmid was confirmed by apramycin resistance and PCR analysis.

3.2.4 Gene expression analysis by semiquantitative RT-PCR

Total RNAs were extracted from mycelia grown in liquid medium B by an RNeasy Mini kit (QIAGEN Science, MD, USA) and treated with DNase I (Takara Bio, Shiga, Japan). The cDNA

was synthesized using SuperScript III RNase H^- reverse transcriptase (Invitrogen) and random primers (Invitrogen) according to the manufacturer's instructions. The cDNAs were amplified from the transcripts of the *far* regulatory genes and the *hrdB* gene using the hrdB-Fw/hrdB-Re primers pair (28). The primers used for the detection of the transcript of the truncated *farR2* gene were tfarR2-Fw and rFarR2-Re. The PCR amplification was performed by using GoTaq Green Master Mix (Promega KK, Tokyo, Japan) under the following conditions: a single round of 95°C for 2 min and discrete cycles (as described in the legend of each figure) of 98°C for 30 s, 65°C for 30 s, and 72°C for 1 min, followed by a single extension of 72°C for 5 min. The PCR annealing temperature for the *hrdB* transcript was 55°C. The absence of DNA contamination was confirmed by RT-PCR without reverse transcriptase. These analyses were performed using total RNAs prepared form two or three independent cultivations to confirm the reproducibility.

3.2.5 Analysis of blue pigment and other secondary metabolites

Culture supernatants were collected periodically and filtrated through 0.2-μm-pore size filters, and the absorbance at 590 nm was measured for the production of blue pigment. The production of D-cycloserine and nucleoside antibiotics was measured as described by Kitani et al. (2001).

3.2.6 Overexpression of *farR2* and *farA* in *E.coli* and protein purification

The *farR2* and *farA* genes were PCR-amplified by the primer pairs rFarR2-Fw/rFarR2-Re and rFarA-Fw/rFarA-Re, respectively. Each fragment was digested with *Nde*I and *Hin*dIII and then cloned in pET-21b digested with the same enzymes, resulting in pLT133 for FarR2 overexpression and pLT134 for FarA overexpression, respectively, which were verified by DNA sequencing. *E. coli* BL21(DE3)/pLysS harboring pLT133 or pLT134 was inoculated into 200 ml of 2xYT medium and the cultivation was continued at 37°C until the optical density at 600 nm

reached 0.6, at which time 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added for induction. After an additional 3 h of cultivation, the collected cells were washed with equal volume of 0.9% NaCl solution, and then resuspended in 10 ml buffer A [50 mM Tris-HCl (pH 7.5) containing 60 mM KCl and 10% glycerol (v/v)]. After sonication and centrifugation (5,000 X g, 5 min, 4^{o}C), the supernatant was loaded onto a Ni Sepharose 6 Fast Flow column (GE Healthcare Bio-Sciences, PA, USA) equilibrated with buffer A. Proteins containing rFarR2 or rFarA were eluted with 5 ml of 100 or 250 mM imidazole (in buffer A), respectively, according to the manufacturer's recommendations. The eluted proteins were dialyzed overnight at 4^{o}C against buffer B [50 mM Tris-HCl (pH 7.5) containing 60 mM KCl and 20% glycerol (v/v)]. Protein concentration was measured with Bio-Rad protein assay kit using bovine plasma gamma globulin as a standard. Purity of rFarR2 or rFarA was analyzed by SDS-PAGE.

3.2.7 PCR conditions for construction and amplification of FITC-labeled probe and gel-shift assay

The fragments were amplified by PCR with the following primer pairs: F31-Fw and F3-Re for F3-1; F32-Fw and F3-Re for F3-2; F33-Fw and F3-Re for F3-3; F34-Fw and F3-Re for F3-4; F11-Fw and F1-Re for F1-1; F12-Fw and F1-Re for F1-2; F13-Fw and F1-Re for F1-3; F21-Fw and F2-Re for F2-1; F22-Fw and F2-Re for F2-2; F23-Fw and F2-Re for F2-3; F24-Fw and F2-Re for F2-4; F41-Fw and F4-Re for F4-1; F42-Fw and F4-Re for F4-2; F43-Fw and F4-Re for F4-3; F41-Fw and F44-Re for F4-4; F41-Fw and F45-Re for F4-5; F46-Fw and F4-Re for F4-6; F61-Fw and F6-Re for F6-1; F62-Fw and F6-Re for F6-2; F63-Fw and F6-Re for F6-3. The amplified fragments were cloned into the *Eco*RV site of pBluescript II SK. The DNA probes were labeled by PCR using these plasmids as templates with an fluorescein isothiocyanate (FITC)-labeled M13-47 primer and RV primer. The FITC-labeled probes (10 ng) were incubated

with 0.5 μg of rFarR2 or rFarA at 25^{o}C for 10 min in 15 μl of DNA binding buffer [25 mM Tris-HCl (pH 7.5), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol (v/v)] containing 1 μg of poly(dI-dC). After incubation, solution was resolved on a non-denaturing 4% (w/v) polyacrylamide gel running for 2 hours (after 30 min pre-run at 100 volt) in buffer containing 50 mM Tris, 380 mM glycine, and 2 mM EDTA, pH 8.5 at the same voltage. Labeled DNA fragments were detected using Typhoon 9210 variable mode imager (GE Healthcare Bio-Sciences).

3.2.8 Determination of transcriptional start site of *farX*

Total RNA was isolated from mycelium harvested at 8 h of cultivation with the exogenous addition of IM-2 at 5 h of cultivation. Transcriptional start site of *farX* was analyzed by a GeneRacer kit (Invitrogen) for rapid amplification of 5' cDNA ends (RACE) as described previously (Miyamoto et al. 2011).

3.3 Results

3.3.1 Features of *farR2*

A phylogenetic tree of FarR2 with other autoregulator receptors and pseudoreceptor regulators in *Streptomyces* indicated that the FarR2 protein clearly belongs to the clade of pseudoreceptor regulators, rather than the clade of autoregulator receptors (Fig. 3.3). These two clades can also be distinguished by their p*I* values: autoregulator receptors have p*I* values of around 5 (p*I* 5.1 for ArpA and p*I* 5.3 for FarA), whereas most of pseudoreceptor regulators show more basic p*I* values (p*I* of 10.2 for BarB and p*I* 7.8 for JadR2). The p*I* value of FarR2 (9.7) is consistent with the results of phylogenetic analysis, implying that FarR2 is most likely to be a pseudoreceptor regulator in *S. lavendulae* FRI-5.

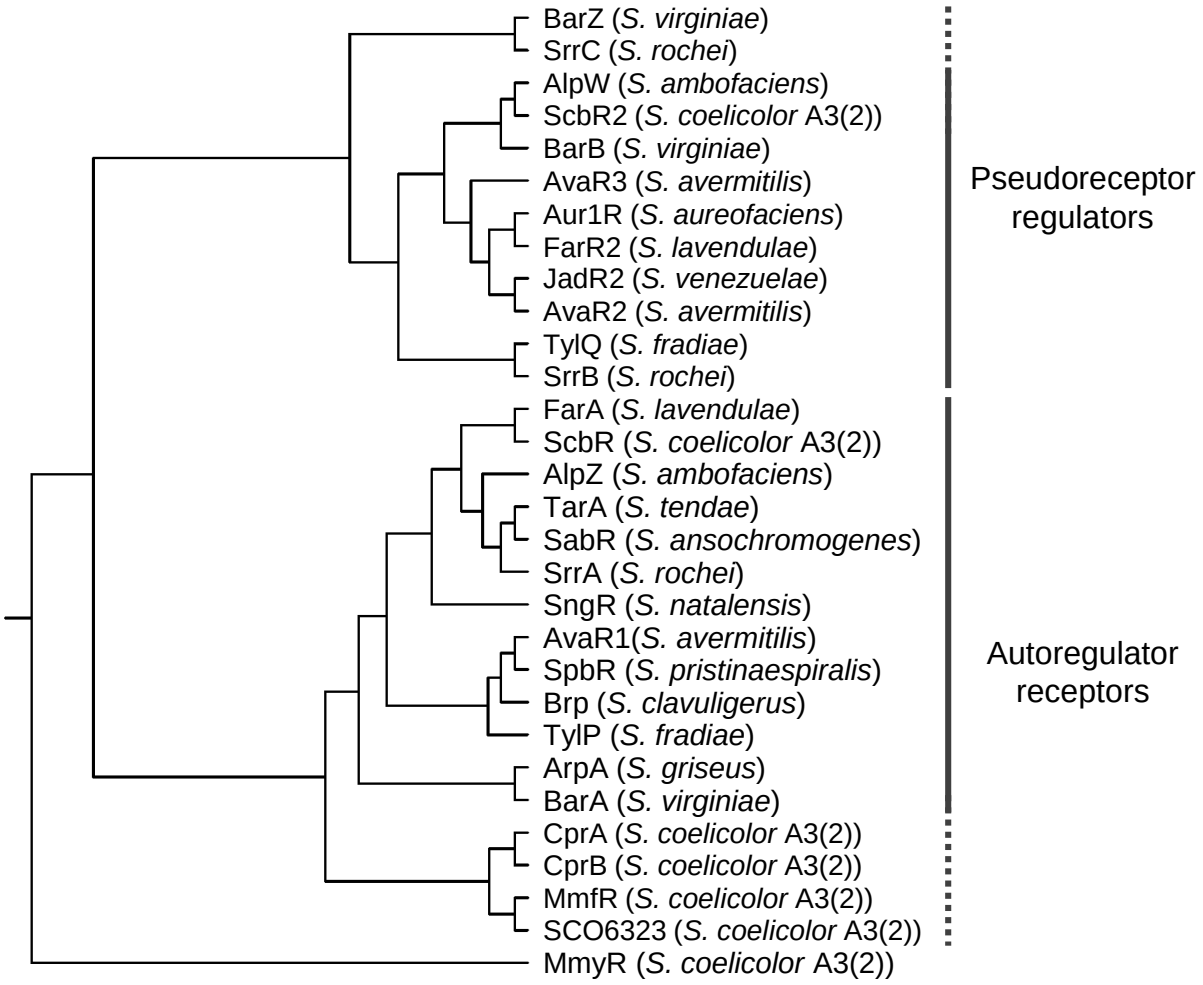


Figure 3.3 Phylogenetic tree of FarR2, pseudoreceptor regulators and autoregulator receptors. Multiple sequence alignment was conducted with the CLUSTALW program (http://www.genome.jp/tools/clustalw/). Phylogenetic trees were constructed by the unweighted-pair group method with the arithmetic mean.

Other than high p*I* values, pseudo-GBL receptor family proteins do not exhibit any GBL binding ability such as in the case of BarB from *S. virginiae* and ScbR2 from *S. coelicolor* (Xu et al. 2010), which were found not to bind virginiae butanolides (GBL in *S. virginiae*) and Scb1 (GBL in *S. coelicolor*), respectively. More detailed analysis of the amino acid sequences

indicated that, although autoregulator receptors contain highly conserved residues Gln and Trp (both important for autoregulator binding) (Sugiyama et al. 1998) and another conserved residue Pro (important for DNA binding) (Onaka et al. 1997), FarR2 has an Ala at the position of Gln and a shorter α7-helix domain with the conserved Trp, similar to those of other pseudoreceptor regulators (Fig. 3.4). These findings suggested that FarR2 may be involved in the regulation of secondary metabolism as a pseudoreceptor regulator responding to secondary metabolites.

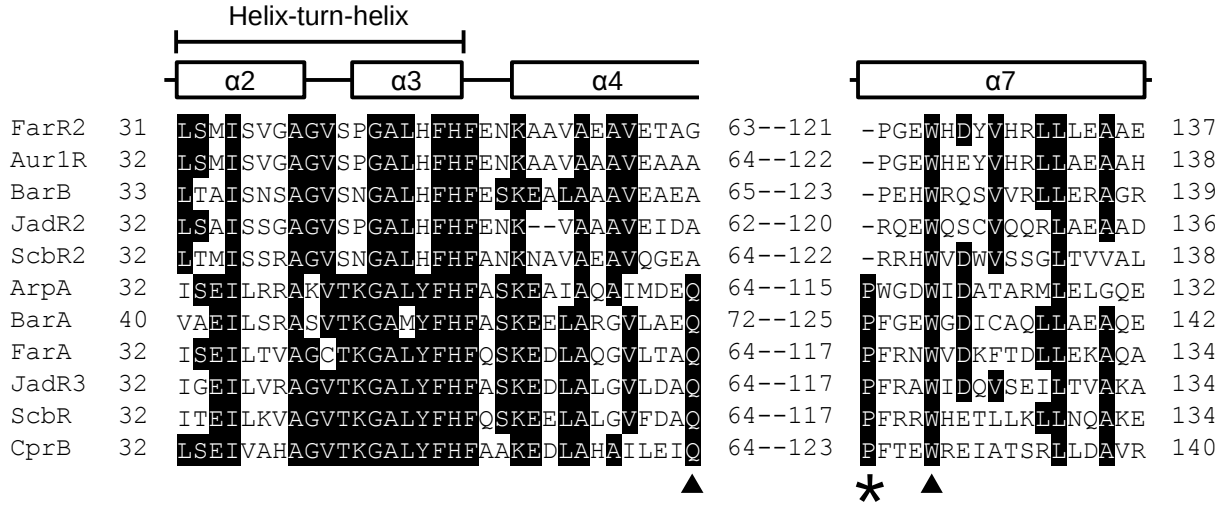


Figure 3.4 Sequence alignment of the regions encoding helix-turn-helix DNA-binding domain, helices α4 and α7 of FarR2 with those of pseudoreceptor regulators and autoregulator receptors. Black boxes indicate positions in the alignment at which the same amino acid is found in at least six of the eleven sequences. The secondary structure elements of CprB are shown above its sequence. Numbers indicate amino acid positions within each sequence. The asterisk and the filled triangles indicate important residues for the binding to DNA and the formation of autoregulator-binding pockets, respectively.

Under normal growth conditions of the wild-type strain, IM-2 concentration reaches a threshold to trigger secondary metabolism at around 8 h of cultivation, and indigoidine production is initiated at 10 h of cultivation (Chapter 1 Fig 1.8). In contrast, external addition of

IM-2 at 5 h of cultivation induces earlier production of indigoidine with growth retardation (Kitani et al. 2001). We previously found by high-resolution S1 nuclease mapping that the transcriptional level of *farR2* is enhanced by the external addition of IM-2 (Kitani et al. 2008), and demonstrated that IM-2 is necessary for the transcription of *farR2* through the analysis of a *farX* disruptant lacking the intrinsic ability to synthesize IM-2 (Kitani et al. 2010). To learn more about the regulation of *farR2* transcription, *farR2* transcription was analyzed in the *farA* disruptant by semiquantitative RT-PCR (Fig. 3.5B). In the wild-type strain, *farR2* transcription was clearly detected at 8 h of cultivation. With the external IM-2, *farR2* was transcribed at 6.5 h but the transcriptional level significantly decreased at 8 h, suggesting that temporal transcription of *farR2* is positively regulated by IM-2. In the *farA* disruptant, regardless of IM-2 addition, constitutive expression of *farR2* was observed, indicating that the *farR2* transcription is negatively controlled by FarA. With the previous finding that FarA binds to the upstream region of *farR2*, these results demonstrated that *farR2* is a direct transcriptional target of FarA *in vivo*, which strongly suggests that FarR2 exert a regulatory function in secondary metabolism governed by the IM-2/FarA system.

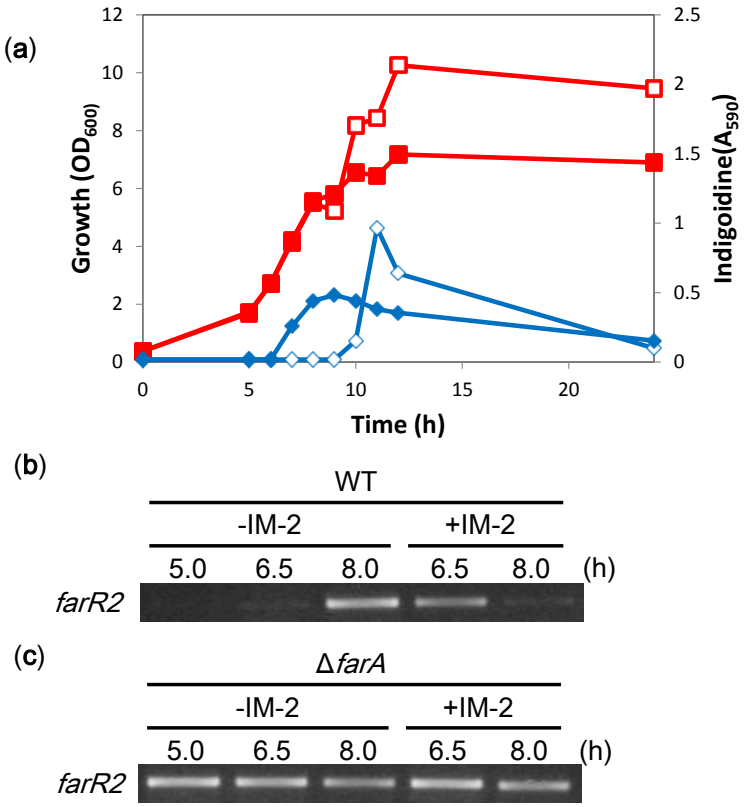


Figure 3.5 Transcriptional regulation on *farR2* by IM-2 and FarA. (A) Growth curves and indigoidine production in the wild-type strain. Growth was monitored by measuring the optical density at 600 nm (OD_{600}). Open-red and filled-red square indicate growth curves without and with the exogenous addition of IM-2 at 5 h of cultivation, respectively. Open-blue and filled-blue diamonds indicate production profiles of indigoidine without and with the addition of IM-2, respectively. (B) Transcriptional profiles of the *farR2* gene by semiquantitative RT-PCR in the wild-type strain (WT) and in the *farA* disruptant (Δ*farA*). Total RNAs were extracted from mycelia harvested at the indicated cultivation times without (-) or with (+) the addition of IM-2.

3.3.2 Effects of *farR2* disruption on the blue pigment indigoidine production in *S. lavendulae* FRI-5

To elucidate the role of *farR2* in the regulation of secondary metabolism, we generated a *farR2* disruptant (Δ*farR2*) by in-frame deletion of 136 amino acids containing the DNA-binding domain and the α7-helix domain. In the absence of external IM-2 addition (Fig. 3.6), the Δ*farR2* strain

showed delayed production of indigoidine with similar growth profile to the wild-type strain.

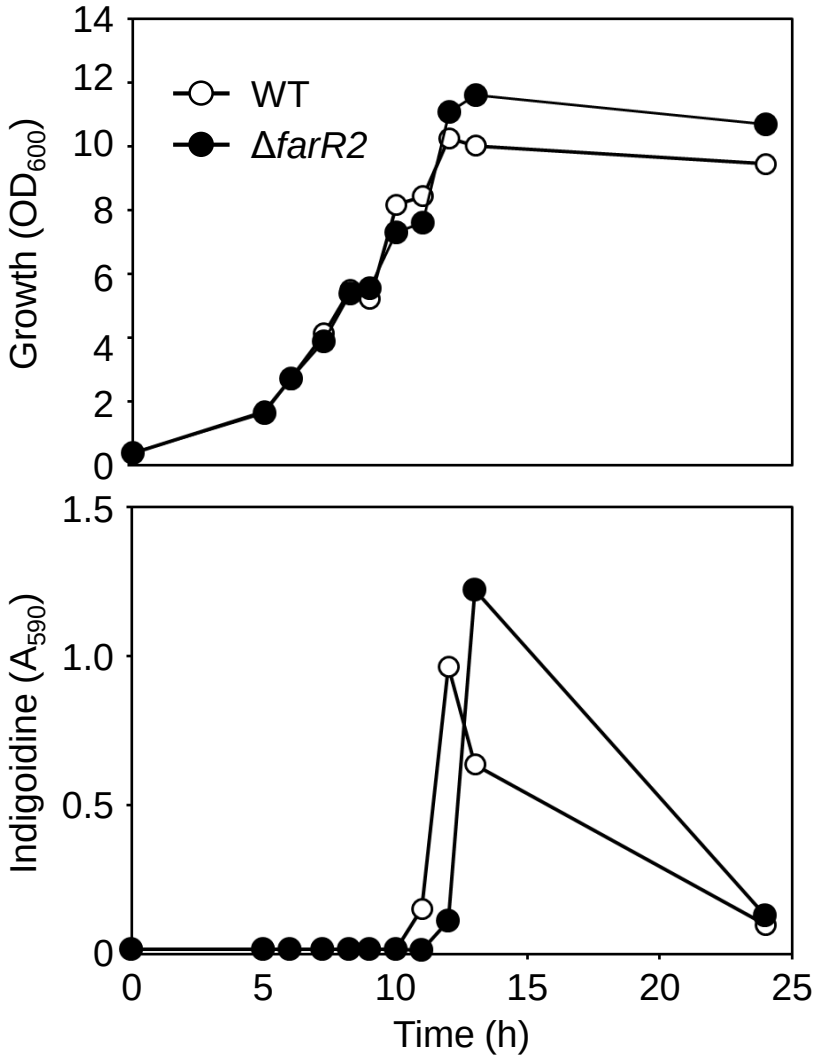


Figure 3.6 Growth curves (A) and indigoidine production (B) in the *farR2* disruptant. WT, wild-type strain (open circles); Δ*farR2*, *farR2* disruptant (filled circles). (A) Growth was monitored by measuring the optical density at 600 nm (OD_{600}). (B) Production profiles of indigoidine in the wild-type strain and in the *farR2* disruptant.

To investigate whether the delayed production of indigoidine is due to delayed biosynthesis of IM-2 or perturbation of IM-2 signaling, IM-2 was externally added at 5 h of cultivation to synchronize the onset of secondary metabolism. The wild-type strain began to produce

indigoidine at 7 h of cultivation, whereas the Δ*farR2* strain did not show any indigoidine production at 7 h but started at 8 h (Fig. 3.7), an 1-h delay identical to that observed without IM-2 addition, indicating that FarR2 does not control the biosynthesis of IM-2 but functions in the regulatory network for indigoidine production. The growth curve of the Δ*farR2* strain was very similar to the wild-type strain (data not shown), and an intact copy of *farR2* into the *farR2* disruptant (Δ*farR2*/*farR2*) restored the production profile of indigoidine to that of the wild-type strain (Fig. 3.7). With respect to the production of D-cycloserine and nucleoside antibiotics, there was no difference between the wild-type strain and the Δ*farR2* strain (data not shown), suggesting that FarR2 is specifically involved in the indigoidine production, and we concluded that FarR2 is a pathway-specific regulator for positively controlling the initiation timing of the indigoidine production.

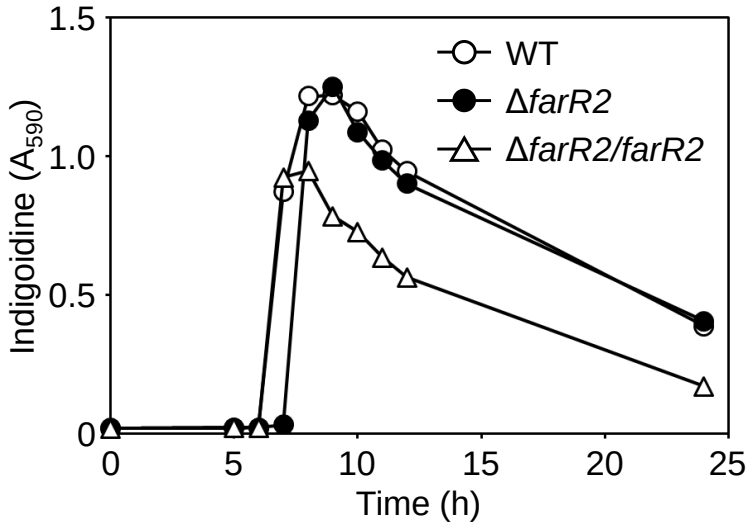


Figure 3.7 Production profiles of indigoidine in the *farR2* disruptant with the addition of exogenous IM-2 at 5 h of cultivation. WT, wild-type strain (open circles); Δ*farR2*, *farR2* disruptant (filled circles); *farR2*-complemented Δ*farR2* strain (open triangles).

3.3.3 Influence of FarR2 on the transcription of *far* regulatory genes

To elucidate the role of *farR2* in the transcriptional regulation of other regulatory genes in the *far*

region, gene expression patterns in the wild-type strain and in *ΔfarR2* were monitored by semiquantitative RT-PCR analysis under the external addition of IM-2 at 5 h, because *farR2* exerts its regulatory function primarily after the perception of IM-2. (Fig. 3.8). All of the investigated regulatory genes in the *far* region were found to be transcribed at low basal levels in the wild-type strain and *ΔfarR2* at 5 h.

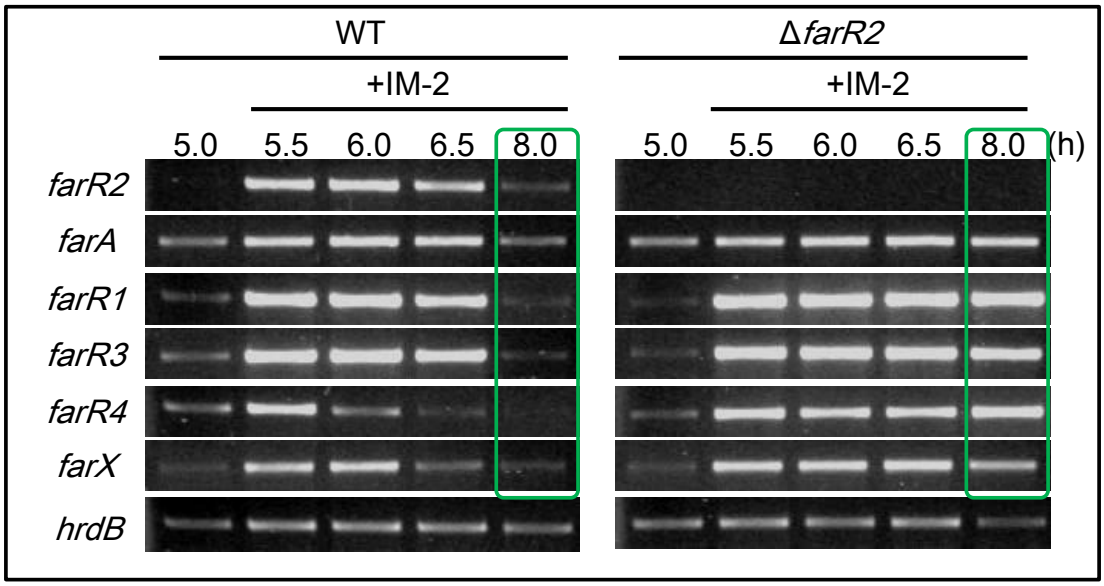


Figure 3.8 Transcriptional analysis of *far*-regulatory genes in the wild-type and*ΔfarR2* strain. Total RNAs were extracted from mycelia harvested at the indicated cultivation times without (-) or with (+) the exogenous addition of IM-2 at 5 h of cultivation. For PCR, 28 cycles of amplification were used for the transcripts of all *far*-regulatory genes and 27 cycles for the *hrdB* transcript. The *hrdB*-like gene [*hrdB* encodes the major sigma factor in *Streptomyces coelicolor* A3(2)] was used as a control, because this gene is expressed fairly constantly throughout growth.

The *farR2* transcript was clearly detected after 5.5 h of cultivation in the wild-type strain and declined at 8 h of cultivation, indicating that the transcription of *farR2* rapidly responds to the IM-2 concentration. Similar pattern of temporal expression was observed for *farA*, *farR1*, and *farR3*, namely increase at the 5.5-h cultivation and decline at the 8.0-h cultivation. However, in the Δ*farR2* strain, the expression at 8 h of these three regulatory genes still remained at the same

levels to those observed at 6.5 h of cultivation. Furthermore, *farR4* and *farX* genes were also expressed at a constant expression level after 6 h and 6.5 h of cultivation, respectively, at which time point the wild-type strain showed the reduced transcription of *farR4* and *farX* compared to that observed at 5.5 h of cultivation. Thus, FarR2 can be concluded to participate in the downregulation of expression for the *far* regulatory genes at the late stage of secondary metabolism activated by the IM-2 signaling cascade.

3.3.4 Binding of FarR2 to FARE3 in the promoter region of *farR2*

Because FarR2 negatively controls the late-stage expression of regulatory genes in the *far* region, it thus seemed possible that FarR2 might regulate its own synthesis. To investigate whether a negative autoregulatory mechanism operates on *farR2*, transcriptional analysis was performed with a pair of primer designed to detect transcript of the region (*farR2**) downstream of the mutation (Fig. 3.9A). In the wild-type strain with the external addition of IM-2, the transcript of *farR2** at 8 h of cultivation was faint, which is consistent with the transcriptional profiles of *farR2* as shown in Fig. 3.5B and Fig. 3.8. However, the Δ*farR2* strain showed increased transcription of *farR2** compared to that of the wild-type strain, indicating that FarR2 negatively functions on the expression of *farR2* itself.

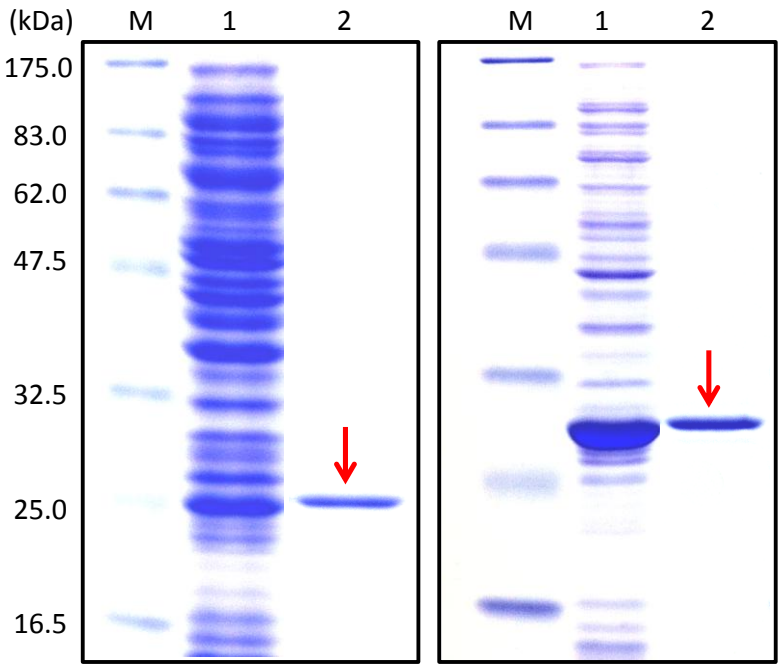


Figure 3.9 SDS-PAGE analysis of rFarR2 and rFarA protein. The arrows indicate the position of target proteins (rFarR2=24.2 kDa; rFarA=25.85 kDa). Lane M: molecular mass marker; Lane 1: crude extract from IPTG-induced *E. coli* BL21(DE3)/pLysS harboring pET *farR2/farA*. Lane 2: purified rFarR2/rFarA.

Recently, it has been reported that a few of pseudoreceptor regulators bind to ARE sequences that are recognized by autoregulator receptors (Wang et al. 2011; Mingyar et al. 2014) and are frequently found in the promoter region of the target genes. FARE3, one of FarA-binding sites, composed of 33-bp sequences including a palindromic structure overlaps with the promoter region of *farR2* (Kitani et al. 2008), suggesting that FarR2 may bind to FARE3 to negatively control its own synthesis. To examine whether FarR2 has DNA-binding activity toward FARE3, we performed a gel shift assay using a purified C-terminal His-tagged FarR2 and DNA fragments encompassing FARE3 (Fig. 3.10B and 3.10C). C-terminal His-tagged FarR2 was produced in *E.coli* BL21/pLysS system and purified as a predominantly single band with an apparent migration at 24.2 kDa (Fig. 3.9 left). Two DNA probes tested, F3-1 and F3-2, including FARE3,

gave a single retarded signal, whereas no retarded signal was observed with a FARE3-deficient probe F3-4, suggesting that FarR2 recognizes FARE3 in the promoter region of *farR2*. To confirm the importance of the imperfect palindromic structure of FARE3, which consists of two conserved hexamers separated by six nucleotides as the FarR2-binding site, we introduced a mutation into the sequence, yielding probe F3-3 showing no significant palindromic structure (Fig. 3.10B).

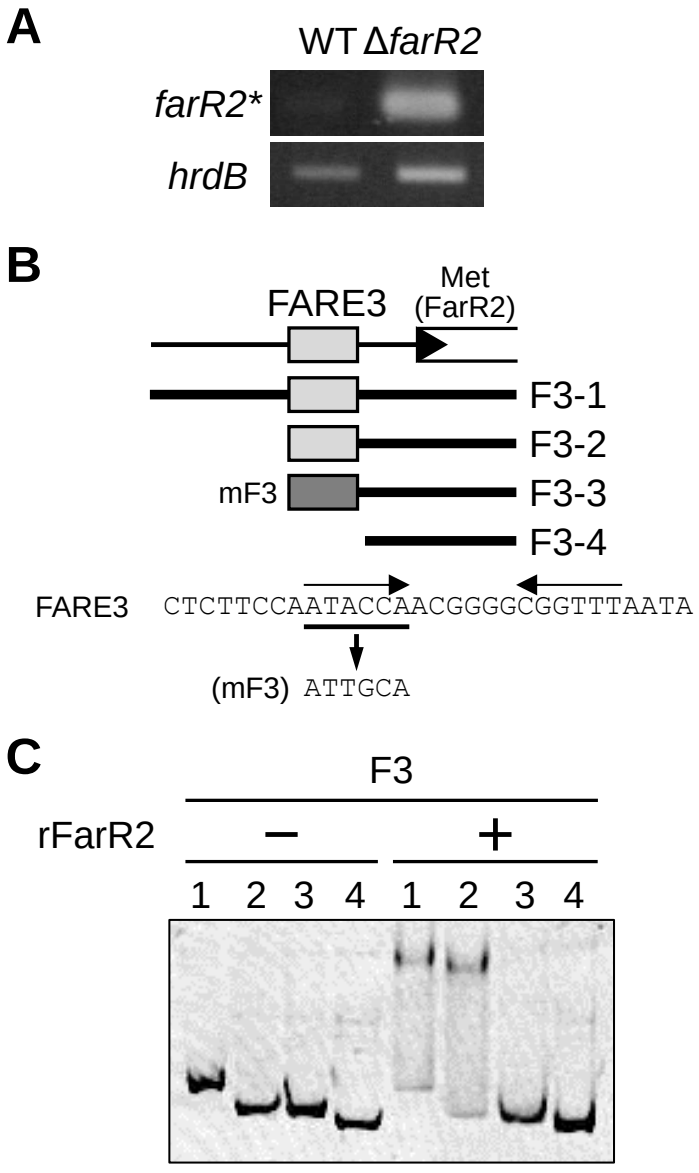


Figure 3.10 Binding of FarR2 to FARE3 located upstream of *farR2*. (A) Transcriptional analysis

of the *farR2* gene in the *farR2* disruptant. WT, wild-type strain; Δ*farR2*, *faR2* disruptant. Total RNAs were isolated from mycelia harvested at 8 h of cultivation with the exogenous addition of IM-2 at 5 h of cultivation. *farR2** indicates the transcript from the truncated *farR2* gene, which is still present in the *farR2* disruptant. PCR of 27 cycles was used for the transcript of the truncated *farR2* genes. (B) Location of probes used for the gel shift assay. The probes F3-1 to F3-4 used in the present study are shown. An arrowhead indicate the translational start codon of FarR2. Arrows above the FARE sequences indicate conserved 6-bp inverted repeats flanking a 6-bp linker region. mF3 represents the mutation introduced by PCR into FARE3. (C) Gel shift assay for the binding of purified His-tagged FarR2 (rFarR2) to probes containing intact or mutated FARE3. The probes F3-1 to F3-4 were incubated in the absence (-) or presence (+) of purified rFarR2.

As shown in Fig. 3.10C, rFarR2 showed no binding to the probe F3-3, demonstrating that the imperfect palindromic structure is required for the binding of FarR2 to FARE3. These findings suggested that FarR2 acts as transcriptional repressor of its own synthesis by binding to its own promoter region.

3.3.5 FarR2 binds to the FAREs located at upstream regions of *far* regulatory genes

The transcriptional analysis in the *farR2* disruptant indicated that the promoter activity of *farA*, *farR1*, *farR3*, *farR4*, and *farX* is negatively regulated by FarR2. The upstream regions of *farA*, *farR1*, and *farR4* contain FARE1, FARE2, and FARE4 (Kitani et al. 2008), respectively, suggesting the possibility that FarR2 recognizes these FAREs to modulate the expression of the three regulatory genes. To further dissect the role of FarR2 in the transcriptional regulation, its ability to bind *in vitro* to the FAREs was investigated using gel shift assays (Fig. 3.11). rFarR2 showed clear binding to DNA probes including FARE1, FARE2, and FARE4, whereas DNA-binding activity of FarR2 was not detected with DNA probes containing mutated FARE or with

FARE-deficient probes. These results indicated that FarR2 directly controls transcription of *farA*, *farR1*, and *farR4*, via binding to their own promoter regions. In contrast, FarR2 showed no binding activity toward the upstream region of *farR3* (data not shown), although this region includes ARE-like sequence, suggesting that transcription of *farR3* may be under indirect control of FarR2.

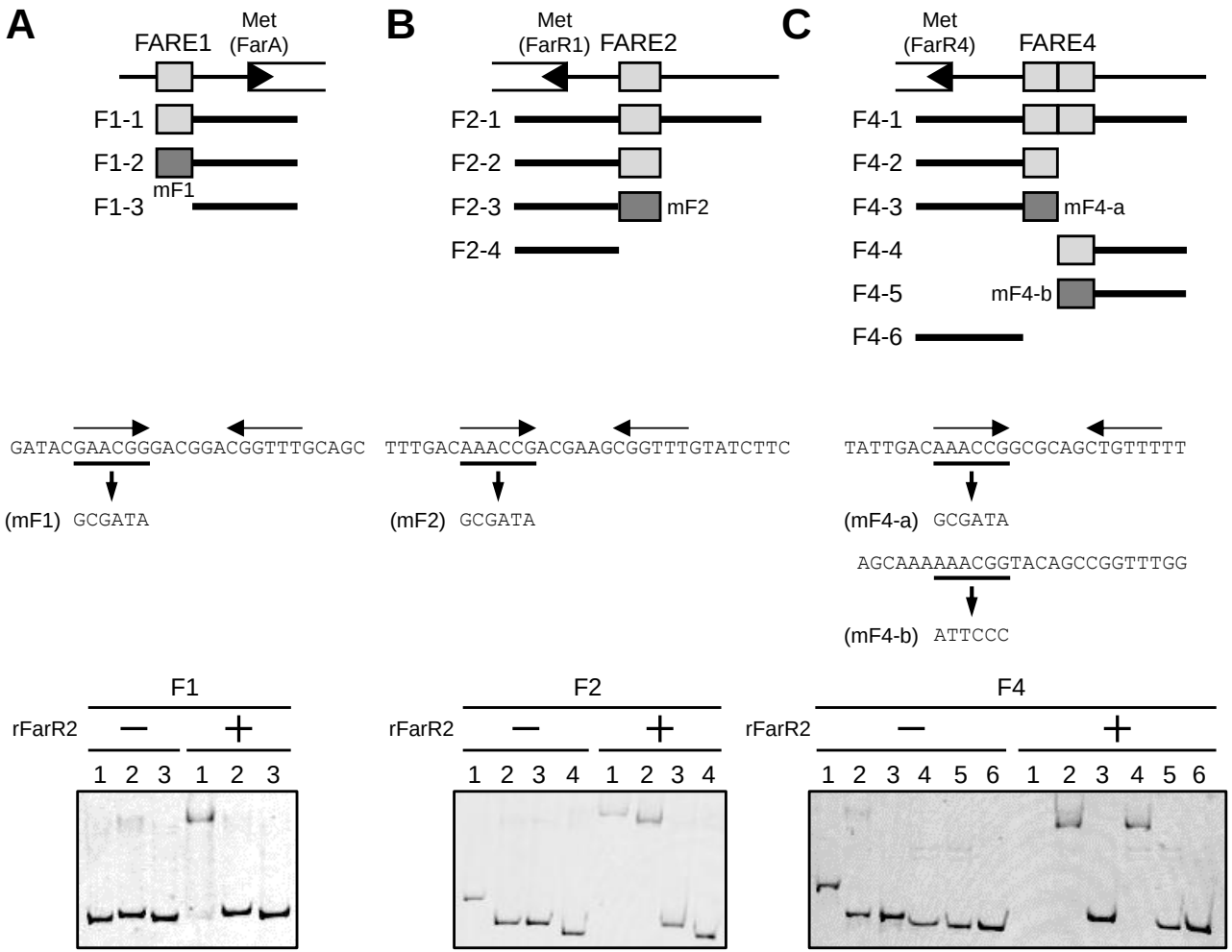


Figure 3.11 Three FAREs (FarA-binding sites) recognized by FarR2. The top panels show locations of probes used for the gel shift assays, the middle panels show intact or mutated FAREs, and the bottom panels show gel shift assays for the binding of rFarR2 to the FARE probes. Arrowheads indicate the translational start codons of FarA, FarR1, and FarR4, respectively. Arrows above the FARE sequences indicate conserved 6-bp inverted repeats flanking a 6-bp linker region. mF1, mF2, mF4-a, and mF4-b represent the mutation introduced by PCR into FARE1 (for mF1), FARE2 (for mF2), and FARE4 (for mF4-a and mF4-b), respectively. Gel shift assays were performed with F1 probes (A), F2 probes (B), and F4 probes (C). The probe was incubated in the absence (-) or presence (+) of rFarR2.

We previously found that a candidate 26-bp ARE-like sequence is present in the 124-bp upstream region of the *farX* gene (Aroonsri et al. 2012), although whether FarA binds to the putative ARE sequence remains unclear. Gel shift analysis demonstrated that rFarR2 specifically binds to this ARE-like sequence, designated FARE6, encompassing an imperfect palindromic structure (Fig. 3.12A). To analyze the promoter region of *farX*, 5'-RACE analysis was performed, which revealed that the transcriptional start site (tss) of *farX* was G, situated 116 nt upstream from the translational start codon of *farX* (Fig. 3.12B). In front of the tss, a possible -10 region was identified, which was similar to the consensus -10 region of streptomycetes Eσ^{70}-like promoters, although no typical -35 region was detected. These results suggested that FarR2 negatively controls the expression of *farX* (the IM-2 biosynthetic gene) by the direct binding to FARE6 in the promoter region of *farX*. To examine the possibility that FarA has DNA-binding activity toward FARE6, a gel shift assay was performed using a purified C-terminal His-tagged FarA (Fig. 3.9 right) and a DNA probe including FARE6 (Fig. 3.12C). Similar to recombinant FarR2 protein, recombinant FarA protein was produced in E. coli BL21/pLysS system and purified as a predominantly single band with an apparent migration at 25.85 kDa (Fig 3.9). A shifted signal was clearly visible with the incubation of rFarA and the probe, but the addition of IM-2 abolished the formation of the FarA-FARE6 complex, suggesting that, like FarR2, FarA might be involved in the direct regulation of IM-2 biosynthesis.

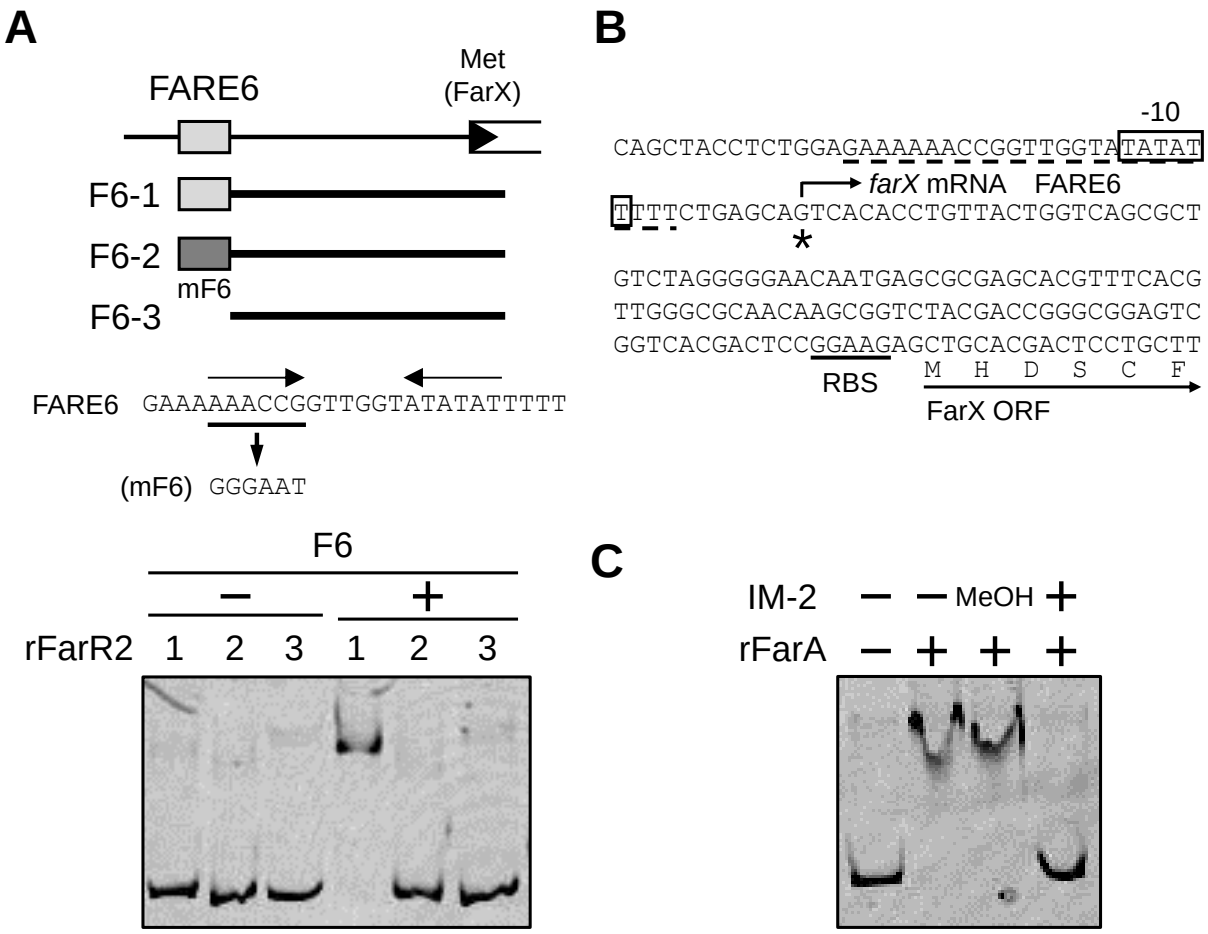


Figure 3.12 Binding of FarR2 to new FarA-binding site located upstream of *farX*. (A) Gel shift assay for the binding of rFarR2 to an ARE-like sequence (FARE6) in the upstream region of *farX*. An arrowhead indicate the translational start codon of FarX. Arrows above the FARE sequences indicate conserved 6-bp inverted repeats flanking a 6-bp linker region. mF6 represents the mutation introduced by PCR into the ARE-like sequence. The probe was incubated in the absence (-) or presence (+) of rFarR2. (B) Nucleotide sequences of the promoter and operator region of *farX*. The asterisk indicate the transcriptional start site of *farX*. The probable -10 region is shown in a box, and the putative ribosome-binding site (RBS) is underlined. The FarA-binding sequence (FARE6), which is bound to FarR2, is indicated with a dashed line. (C) Gel shift assay for the binding of rFarA to FARE6. When IM-2 was added to the reaction mixture at a final concentration of 350 nM, the mixture was incubated at 25^{o}C for a further 5 min before the gel shift assay was performed. As a negative control, MeOH, which was used to dissolve IM-2, was added to the reaction mixture.

3.4 Discussion

In *Streptomyces* sp., the GBL autoregulator-receptor pair that mediates regulation is widely employed to control the production of secondary metabolites and/or morphological differentiation. In our previous studies, we successfully unraveled some components of the GBL autoregulator signaling cascades in *S. lavendulae* FRI-5. In this study, I demonstrated that FarR2 (a pseudoreceptor regulator) is under the tight and direct transcriptional control of the IM-2/FarA system, and revealed that FarR2 is acting as the pathway-specific activator on the onset of indigoidine production as well as the direct repressor of the *far* regulatory genes including *farR2* itself at the late stage of secondary metabolism. These findings suggest that FarR2 has distinct contributions to two physiological processes in the different stages of secondary metabolism.

In chapter 2, I demonstrated that FarR3, a member of the SARP family regulators locating in the *far* regulatory island positively controls not only the amount of indigoidine but also the onset of the indigoidine production (Kurniawan et al. 2014), implying that two different types of regulators (FarR3 as a SARP family regulator and FarR2 as a pseudoreceptor regulator) in the IM-2/FarA system have redundant functions in the regulation of indigoidine production. At the beginning stage of secondary metabolism activated by IM-2, lack of *farR2* did not show any effect on the expression of *farR3* (Fig. 4) and vice versa (Kurniawan et al. 2014), thus indicating that two independent pathways in the IM-2/FarA regulatory cascade governs the onset of indigoidine production.

In clear contrast to the positive effect of FarR2 on the onset of indigoidine production, the transcriptional analysis of *far*-regulatory genes in the *farR2* disruptant indicated that FarR2 acts as a transcriptional repressor. As is the case with true GBL receptors, with pseudo-GBL receptor genes the product mediates the transcriptional effects on the target genes by binding to a specific sequence in the promoter region of each target genes. Many of the binding sequences of

pseudo-GBL receptors share high degrees of similarity with the binding sequences of their respective true GBL receptors. FarR2 demonstrated direct binding to FARE3 located upstream *farR2*, as observed through in vitro analysis of purified recombinant FarR2 in gel-retardation assays. Taken together with the transcription of *farR2* in the *farR2* disruptant, suggest that FarR2, similar to FarA, forms an autoregulatory circuit to control its own expression. The extensive gel shift assays also demonstrated that FarR2 recognizes FAREs locating at the promoter regions of the *far* regulatory genes that are transcriptionally repressed by FarR2. We have already found that a putative indigoidine biosynthetic gene [significant identity (98%) with BpsA from *S. lavendulae* ATCC11924, which is involved in the biosynthesis of indigoidine] is present in the left-hand region approximately 21 kb away from the *farR2* locus (our unpublished data). However, no ARE-like sequence, characterized by two conserved hexamers forming a palindromic structure split by six nucleotides (5`-AWACSG-$N_6$-CBGTTT-3`), is found in the upstream region of the putative indigoidine biosynthetic gene. The imperfect palindromic sequence are always found in the upstream region of target genes of the true GBL and pseudo-GBL receptors family, suggesting the importance of the palindromic sequence for recognition by the DNA binding domain of the receptors (Folcher et al. 2001). Therefore, it is unlikely that FarR2 might recognize a region distinct from the FAREs conserved sequence, as my data (Fig. 3.10-12) demonstrated that the palindrome sequences in the FAREs are essential for recognition by FarR2. Thus, taken together with the observation that the pseudoreceptor regulators act mainly as transcriptional repressors, we suggest that FarR2 operates via another regulator to positively regulate the indigoidine biosynthesis.

Transcriptional upregulation of the majority of *far*-regulatory genes, in the *farR2* disruptant occurred after the production of indigoidine was initiated at the late exponential phase, indicating that the inhibition activity of FarR2 in the wild-type strain occurs after the late

exponential phase, in the entry into the stationary phase. Transcriptional repression of *far*-regulatory genes at the stationary phase may lead to the termination of IM-2 production.

In previous studies, the DNA binding activity of pseudo-GBL receptors is not inhibited by the GBL signaling molecule, which is an important characteristic of these so-called pseudo-GBL receptors. This is true for FarR2 because IM-2 showed no inhibitory activity on the formation of the FarR2-DNA complex (Fig. 3.13).

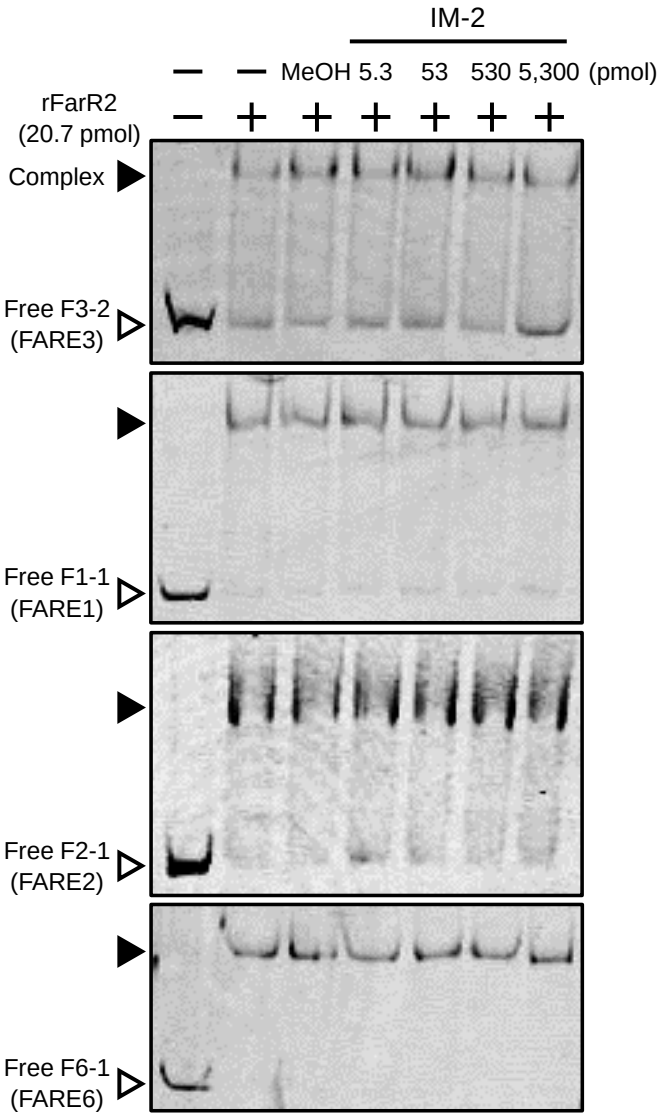


Figure 3.13 Effect of IM-2 on DNA-binding activity of FarR2. When IM-2 was added to the

reaction mixture, the mixture was incubated at 25ºC for 5 min. As a negative control, MeOH, which was used to dissolve IM-2, was added to the reaction mixture. A black triangle indicates the position of the rFarR2-DNA complex, and a white triangle indicates the position of the probe DNA only

Previous reports demonstrated that secondary metabolites, production of which is controlled by these regulators, control the activity of pseudo-GBL receptors through binding to the ligand-binding domain. FarR2 has a specific DNA-binding activity to 5 FAREs (FARE1, FARE2, FARE3, FARE4, and FARE6) in the promoter regions of the *far* regulatory genes. However, indigoidine did not affect the DNA-binding activity of FarR2 (Fig. 3.14). The *far* regulatory genes that are under the control of FarR2 are found to be transcribed in a growth-dependent manner (Fig. 3.8, left panel), suggesting that effective ligand (rather than IM-2 nor indigoidine) of FarR2 for affecting the DNA-binding activity might be present for modulating the expression of those genes.

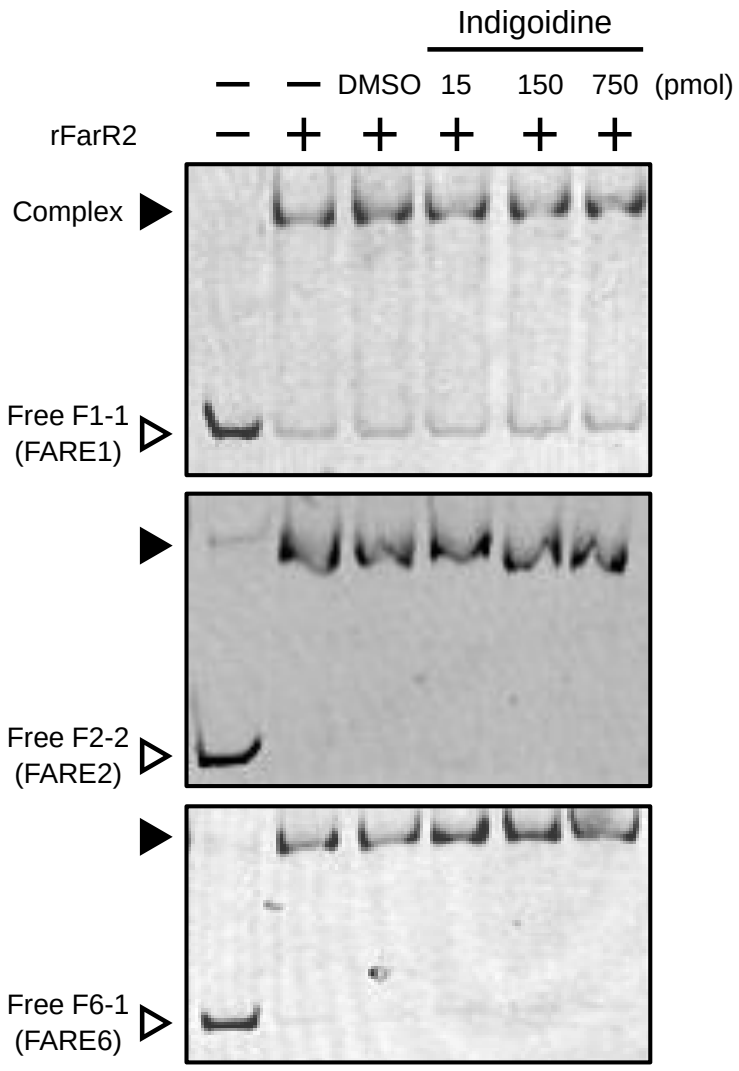


Figure 3.14 Effect of indigoidine on DNA-binding activity of FarR2. When indigoidine was added to the reaction mixture, the mixture was incubated at 25$_{o}$C for 5 min. As a negative control, DMSO, which was used to dissolve indigoidine, was added to the reaction mixture. A black triangle indicates the position of the rFarR2-DNA complex, and a white triangle indicates the position of the probe DNA only.

In the absence of IM-2, FarA represses the transcription of *farR2* via binding to FARE2, and the binding of IM-2 to DNA-bound FarA allows *farR2* to be upregulated. Thereafter, FarR2 binds to FARE1 in the promoter region of *farA* to downregulate *farA* expression at the late stage of secondary metabolism, indicating a negative feedback mechanism to achieve adequate expression.

Intriguingly, the expression of *farX*, an IM-2 biosynthetic gene, is also negatively regulated by FarR2. IM-2 production is prominently observed in the late exponential phase and declined gradually in a growth-dependent manner (Yanagimoto et al. 1988). Because the production profile of IM-2 is almost similar to the expression profile of *farX*, the decreased production of IM-2 at the stationary phase can occur by the binding of FarR2 to FARE6 in the promoter region of *farX* to suppress its transcription. In *S. coelicolor* A3(2) and in *S. venezuelae*, ScbR2 and JadR2 bind to ARE-sequences in the promoter regions of the γ-butyrolactone biosynthetic genes for repressing the biosynthesis of the γ-butyrolactone autoregulators, respectively (Wang et al. 2011). Therefore, negative transcriptional regulation for γ-butyrolactone biosynthesis appears to be a common feature of the pseudoreceptor regulators.

The function of the pseudoreceptor regulators has been gradually unveiled in a few of *Streptomyces* species. JadR2 and Aur1R repress the production of jadomycin and auricin whose biosynthetic gene clusters include the *jadR2* and *aur1R* gene, respectively, and the cognate antibiotic shows an inhibitory activity toward the formation of the pseudoreceptor regulator-DNA complex (Xu et al. 2010; Novakova et al. 2010). On the other hand, ScbR2 locating in the biosynthetic gene cluster of coelimycin plays a negative role in the production of coelimycin, and positively regulates the production of another antibiotic such as actinorhodin (Act) and undecylprodigiosin (Red) (Xu et al. 2010). Unlike the case of JadR2 and Aur1R, *scbR2* locates far distal from the biosynthetic gene cluster of Act and Red, but Act and Red still affect the DNA-binding activity of ScbR2 for the regulation of antibiotic production. Our findings demonstrated that FarR2 facilitates the initiation timing for the production of indigoidine without controlling the yield of indigoidine, and thus FarR2 is regarded as an activator-type pseudoreceptor regulator for secondary metabolism. Moreover, it is of interest that indigoidine has no function for the formation of the FarR2-FARE complex. Further understanding of the

regulatory mechanism of FarR2 together with the identification of the FarR2-ligand will provide greater insights into common or unique features of the pseudoreceptor regulators in the regulation of secondary metabolism in streptomycetes.

3.5 Summary

The γ-butyrolactone autoregulator signaling cascade distributes widely among many *Streptomyces* species as an important regulatory system of secondary metabolism. The pseudoreceptor regulator, although highly homologous to the autoregulator receptor, has different mode of function in the regulation of secondary metabolism from that of the autoregulator receptor. In *Streptomyces lavendulae* FRI-5, a γ-butyrolactone autoregulator IM-2 and the IM-2 specific receptor FarA control the production of blue pigment indigoidine together with two types of antibiotics: D-cycloserine and the nucleoside antibiotics. Here, we demonstrated that *farR2* (a *farA* homologue) locating in the clustered regulatory genes including *farA* is classified as a gene of the pseudoreceptor regulator family by *in silico* analysis, and that the expression of *farR2* is controlled by the IM-2/FarA regulatory system. Disruption of *farR2* resulted in delayed production of indigoidine and in transcriptional derepression of the clustered *far* regulatory genes. Furthermore, FarR2 binds to the FarA-binding sequences in the promoter regions of the regulatory genes which are downregulated by FarR2. These findings suggested that FarR2 acts as a pleiotropic regulator that controls secondary metabolism under the IM-2 signaling cascade.

Chapter 4

Conclusion

Members of the Gram-positive, soil-dwelling filamentous bacterial genus *Streptomyces* have been extensively studied due to their complex life cycle of morphological differentiation, their ability to synthesize secondary metabolites with structural and biological diversity, and their secondary metabolites' medical and industrial significance. The production of these secondary metabolites is tightly regulated in a hierarchical manner with several layers involving higher-level regulators regarded as global regulators that mediate stimuli from the environment, controlling the activity of the low-level pathway-specific regulators that directly control the activation of biosynthetic genes for secondary metabolites.

In *Streptomyces*, the most well-known hierarchical regulation is the γ-butyrolactone signaling cascade consisting of a γ-butyrolactone molecule and a cognate γ-butyrolactone receptor protein which is situated at the highest level of the hierarchy in the regulatory cascade (Folcher et al. 2001; Bibb 2005). In the absence of γ-butyrolactone, the γ-butyrolactone receptor protein binds to a specific DNA sequence in the promoter region of target genes and represses its transcription. When the γ-butyrolactone molecule is produced and reaches a critical threshold concentration, it binds the DNA-bound receptor and leads to the dissociation of the receptor protein from the promoter of target genes, thereby triggering the transcriptional activation of target genes and allowing the onset of secondary metabolism and/or morphological development.

The γ-butyrolactone signaling cascade in *Streptomyces lavendulae* FRI 5 is composed of a γ-butyrolactone molecule, IM-2, and the cognate γ-butyrolactone receptor FarA. Unlike other γ-butyrolactone molecules which usually play only positive roles in the regulation of secondary

metabolite production, IM-2 exerts both positive and negative effects on the regulation of secondary metabolism; namely, it switches on the production of blue pigment and nucleoside antibiotics and switches off the production of D-cycloserine. Although the function of the receptor protein FarA and IM-2 have been extensively studied and a model of their signaling cascade was created, there are still missing links regarding how they actually play their roles in the production of secondary metabolites. We previously found that the *farA*-flanking region has seven regulatory genes (including *farX*, an IM-2 biosynthetic gene) and comprises a *far* regulatory island (Kitani et al. 2008, 2010). Two putative regulatory genes (*farR3* and *farR4*) encoding the *Streptomyces* antibiotic regulatory protein (SARP) family proteins are present in the *far* regulatory island together with two more putative transcriptional regulatory genes (*farR1* and *farR2*), all of which are considered to be the direct transcriptional targets of FarA and therefore might be involved in the IM-2/FarA signaling cascade.

In this dissertation, I focused on three regulatory genes in the *far*-regulatory island presumably involved in the IM-2/FarA signaling cascade.

In Chapter 2, I characterized two regulatory genes of the SARP family, *farR3* and *farR4*, which are tandemly located in the proximal region of *farA*. The SARP family regulators are DNA-binding proteins transcriptional regulators and in general act as activators for the production of secondary metabolites. *farR3* is transcribed as both a monocistronic RNA and a bicistronic *farR4-farR3* mRNA, and the expression profile is tightly controlled by the IM-2/FarA system. Loss of *farR3* delayed and decreased the production of blue pigment without any changes in the transcriptional profile of other *far* regulatory genes, indicating that FarR3 positively controls the biosynthesis of blue pigment and is positioned in the downstream region of the IM-2/FarA signaling cascade.

The blue pigment was later identified as indigoidine, a pigment synthesized by a single

module non-ribosomal peptide synthetase (NRPS). Loss of *farR4* induced the early production of IM-2 by increasing the transcription of an IM-2 biosynthetic gene, *farX*, indicating that FarR4 negatively controls the biosynthesis of IM-2 temporally. Taken together with the model proposed previously for a negative autoregulatory circuit for IM-2 production at the stationary phase, the present findings indicate that FarR4 is an important determinant for controlling the initiation time of IM-2 production.

This is the first report to show that a SARP-family regulator is involved in the biosynthesis of a signaling molecule functioning at the most upstream region of the regulatory cascade for *Streptomyces* secondary metabolism. Thus, my results suggest differing contributions of the SARP-family regulators to the regulation of indigoidine production in *S. lavendulae* FRI-5.

In Chapter 3, I characterized a regulatory gene of the γ-butyrolactone receptor homologue family, *farR2*, which is located downstream of *farR3*. Due to the high p*I* value, FarR2 falls into the subclass of pseudo-γ-butyrolactone receptors. In general, pseudo-γ-butyrolactone receptors negatively control the production of secondary metabolites. Similar to *farR3* and *farR4*, the transcription of *farR2* is tightly controlled by the IM-2/FarA system. Loss of *farR2* delayed the production of indigoidine, indicating a function similar to that of *farR3* to positively control indigoidine production.

In clear contrast of the delayed effect on indigoidine production, loss of *farR2* caused the transcriptional upregulation of *far*-regulatory genes, indicating that FarR2 acts as a transcriptional repressor. The in-vitro analysis demonstrated that FarR2 binds to the FAREs located at upstream regions of *far* regulatory genes The transcriptional upregulation of the majority of *far*-regulatory genes in the *farR2* disruptant occurred after the indigoidine production reached a maximum at the late exponential phase, indicating that FarR2 elicits its transcriptional inhibition in the later phase of indigoidine production, possibly in the transition phase between the late exponential phase and

the stationary phase. Transcriptional inhibition at this phase may lead to the termination of IM-2 signaling cascade.

Taken together with the positive effect on the onset of indigoidine production, the present findings demonstrate that FarR2 makes distinct contributions to two physiological processes in the different stages of secondary metabolism.

All results presented here showed that the three newly characterized genes *farR3*, *farR4*, and *farR2* are essential in the temporal regulation of IM-2 and indigoidine production. Lastly, although it is not easy to present a simple model for the regulation of indigoidine production and IM-2 biosynthesis, I constructed a new working model (Fig. 4.1) divided into 3 major phases with 4 sub-phases as follows by taking into account all observations described in this dissertation and our previous publications.

Pre-activation phase, consist of only one sub-phase:

Phase 1: At the early stages of growth before IM-2 production, the basal level transcription of *farA* provides sufficient ligand-free FarA to repress its own gene by binding to the FARE site upstream of *farA*, forming an autoregulatory circuit. At the same time, FarA represses the transcription of *farR2* by binding to FARE3 and *farR3*, which are important for the onset and control of indigoidine production. IM-2 production is not initiated due to the repression of the IM-2 biosynthetic gene *farX* by the SARP-family regulator FarR4.

Activation phase, consist of two sub-phases:

Phase 2: At the middle of the exponential phase, the expression of *farR3* increases gradually by an unknown mechanism while *farR2* transcription is still repressed by FarA. FarA activates *farX* transcription to initiate IM-2 biosynthesis, possibly by overriding the repressing activity of FarR4 to *farX*.

Phase 3: When the IM-2 concentration reaches a threshold, it forms a complex with FarA, leading

to the dissociation of the IM-2-FarA complex from FARE3, allowing *farR2* transcription to be initiated. Together with FarR3, FarR2 consequently initiates indigoidine production.

Post-activation phase, consist of only one sub-phase

Phase 4: The termination process. After indigoidine production reaches a maximum, free FarR2 binds to the unoccupied FARE3 to repress its own gene transcription, leading to the initial step in the termination of IM-2 and indigoidine biosynthesis. At the same time, FarR2 directly binds and represses transcription of *far*-regulatory genes, including *farX*, leading to the complete termination of IM-2 signaling cascade.

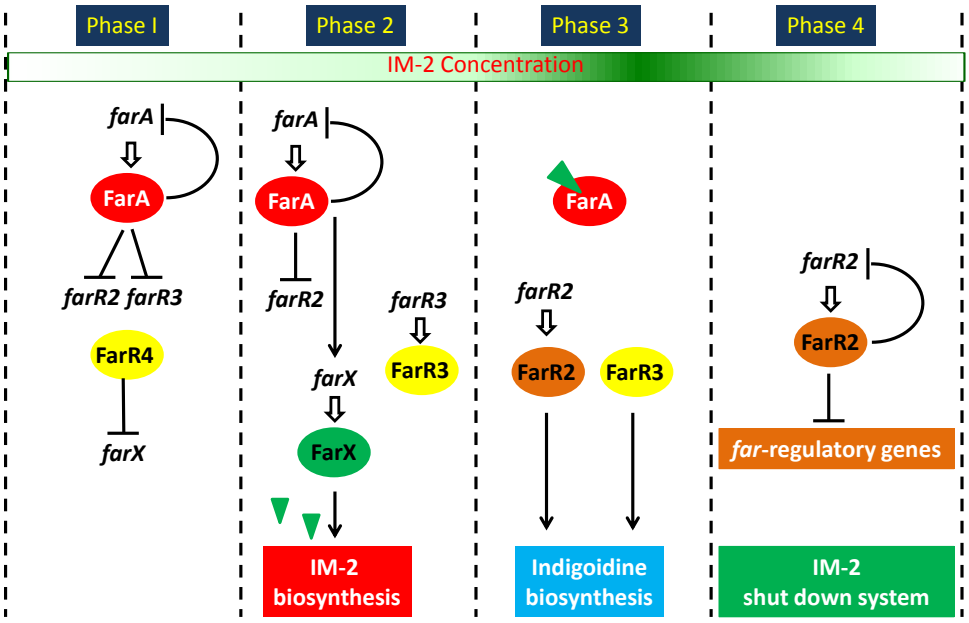


Figure 4.1 A new model depicting the IM-2/FarA signaling cascade for the regulation of the production of IM-2 and the blue pigment indigoidine.

Overall, the new model demonstrates that the temporal regulation of IM-2 and indigoidine production is very exquisite and finely tuned. Further knowledge of how IM-2/FarA controls the production of the remaining secondary metabolites will clarify the regulatory network for secondary metabolism in *Streptomyces*.

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