DOCTORAL DISSERTATION

METABOLIC ENGINEERING OF ACTINOMYCETES: STUDIES TO IMPROVE AND ACTIVATE POLYKETIDES PRODUCTION FROM *Streptomyces* AND *Nocardia species*

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ACTINOMYCETES 의 대사공학 연구: *Streptomyces* 와 *Nocardia* 균주로부터 POLYKETIDE 의 생산성 증가를 위한 연구

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(Abstract)

Metabolic Engineering of Actinomycetes: Studies to improve and activate polyketide production from *Streptomyces* and *Nocardia species*

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Metabolic engineering approaches have been employed intensively to improve the production of therapeutically important polyketides, including manipulation of native producers and development of heterologous host. Similar molecular biologic techniques, improving flux through the biosynthetic pathway, enhancing precursor supply and reducing by-product formation, are used for native and heterologous hosts. There are many successful examples of manipulation in native producers and use of heterologous hosts to improve the production of bioactive polyketides.

In this study, we focused on these metabolic engineering approaches to improve homologous and heterologous production of bioactive secondary metabolites from *Streptomyces* and *Nocardia* species.

With the aim to enhance the production of pikromycin from native producer, two positive regulator genes, *metK1-sp* and *afsR-sp*, which encode for the MetK protein and global regulatory protein respectively from *S. peucetius* ATCC 27952 were heterologously expressed in *S. venezuelae* ATCC 15439. The production of pikromycin was increased by 1.6-fold and 2.6-fold by the expression of *metK1-sp* and *afsR-sp* respectively. The overexpression of *metK1-sp* and *afsR-sp* in *S. venezuelae* stimulated the expression of the pathway-specific regulatory gene (*pikD*) and ketosynthase gene (*KS*) as demonstrated by RT-PCR. The elevated transcripts of the *pikD* and *KS* genes were consistent with the enhanced production of pikromycin (Chapter III).

During our genome analysis study, we obtained 3.1kb open reading frame (ORF), encoding global regulatory gene *afsR,* from *S. venezuelae* ATCC 15439. It is designated as *afsR-sv*. The deduced product of *afsR-sv* (1,056 aa) was found to have high homology with the global regulatory protein AfsR. Homology-based analysis showed that *afsR-sv* represents a transcriptional activator belonging to the *Streptomyces* antibiotic regulatory protein (SARP) family that consists of N-terminal SARP domain containing a bacterial transcriptional activation domain (BTAD), an NB-ARC domain, and a C- terminal tetratricopeptide repeat domain. We used this global regulatory gene to enhance the production of different therapeutically important polyketides in native producers. The *afsR-*

sv overexpressed strain, *S. venezuelae*/pASV152 exhibited an approximately 4.85-fold increase in pikromycin production when compared to wild-type *S. venezuelae*. Gene expression analysis by RT-PCR demonstrated the activation of transcription of genes belonging to pikromycin production, when *afsR-sv* was overexpressed in *S. venezuelae*. Similarly, heterologous expression of the *afsR-sv* in different *Streptomyces* strains resulted in increased production of the respective antibiotics, suggesting that *afsR-sv* is a positive regulator of antibiotics biosynthesis. The amount of doxorubicin produced by *S. peucetius*/pASV25 was approximately 8 times greater than that produced by the wild-type strain and the amount of actinorhodin produced by *S. lividans* TK24/pASV25 was approximately 1.5 times greater than that produced by the wild-type strain (Chapter IV).

After the successful experiments of manipulation in native producers to improve the production of secondary metabolites, we aimed to develop the efficient heterologous host since many bioactive secondary metabolite producing strains are difficult to cultivate, grow slowly and some microorganisms are not possible to engineer genetically. Also in some cases because of the complexities of pathway networks in microorganisms, it is difficult to obtain desired results by manipulating multiple regulators.

Using metabolic engineering approach, we developed *S. venezuelae* YJ028 as an efficient heterologous host so as to increase the precursor pool to be directed towards enhanced production of various polyketides. We engineered *S. venezuelae* YJ028 by expressing acetyl-CoA carboxylase (ACC), propionyl-CoA carboxylase (PCC), *metK1-sp* and *afsR-sp* separately generating four different *S. venezuelae* YJ028 mutant hosts. To probe

the applicability of newly developed hosts in the heterologous production of polyketides, we expressed type III polyketide synthase, 1,3,6,8-tetrahydroxynaphthalene synthase. Flaviolin production was doubled by expression of ACC and 4-fold by combined expression of ACC, *metK1-sp* and *afsR-sp*. Thus, the newly developed *S. venezuelae* YJ028 hosts produce heterologous polyketides more efficiently than the parent strain (Chapter V).

With the target of mass production of eco-friendly herbicide and also to generate novel analogues of herboxidiene, we carried out the metabolic engineering of herboxidiene producer, *S. chromofuscus* ATCC 49982 via expression of ACC and PCC together with methyl group donor gene, *metK1-sp* and global regulatory gene, *afsR*-p01. After expression of these genes with the abolishment of herboxidiene production, all *S. chromofuscus* mutant strains showed change in morphology and produced blue and red pigmented compounds irrespective of the genes expressed. From complete analyses of blue and one of the red compounds, we found that all these strains produced blue pigment antibiotic, actinorhodin and red colored immunosuppressive drug, undecylprodigiosin. (Chapter VI).

To enhance production of nargenicin A1 in *Nocardia* sp. CS682, we carried out expression of *metK1-sp* and ACC individually and also together with proline feeding. When only *metK1-sp* was expressed, nargenicin was found to be enhanced by 2.8 times in *Nocardia* sp. metK18 as compared to that of *Nocardia* sp. CS682 or *Nocardia* sp. NV18. When ACC was expressed, we found that the production of nargenicin in *Nocardia* sp. ACC18 was increased by 3.85 times than that produced by *Nocardia* sp. CS682 or *Nocardia* sp. NV18. But unexpectedly, when 3.0 g/L proline was fed, the production of nargenicin was

decreased by about 50% in *Nocardia* sp. ACC18 as compared to wild type without proline feeding. Every time when we isolated and analyzed compounds from *Nocardia* sp. ACC18, with or without feeding proline, we observed that a peak corresponding to another compound was found to be increased much more than nargenicin as revealed by HPLC analyses. Purification and analysis of the compound by LC/MS, ^{1}H-NMR, ^{13}C-NMR and COSY revealed that the compound was isonargenicin. Thus, we concluded that the production of nargenicin is stimulated by expression of *metK1-sp* and ACC and inhibited by proline feeding. Instead, we observed that the production of isonargenicin was enhanced much more when ACC was expressed and proline was fed as compared to nargenicin (Chapter VII).

Key words: Metabolic engineering, *metK1-sp, afsR-sp,* acetyl-CoA carboxylase, propionyl-CoA carboxylase, *S. venezuelae* YJ028, pikromycin, *S. chromofuscus* ATCC 49982, herboxidiene, actinorhodin, undecylprodigiosin, *Nocardia* sp. CS682, nargenicin

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Abbreviations

aa	amino acid
bp	base pair
ACP	acyl carrier protein
A. erythreum	*Aeromicrobium erythreum*
ACC	acetyl-CoA carboxylase
ARO	aromatase
ATCC	American type culture collection
ATP	adenosine-5'-triphosphate
BHI	brain heart infusion
1,3-BPG	1,3-diphosphoglycerate
CoA	coenzyme A
CYC	cyclase
cm	centimeter
13C-NMR	carbon-13 nuclear magnetic resonance
COSY	correlation spectroscopy
DOSY	Diffusion-Ordered NMR Spectroscopy
DevB	phosphoglucolactonase
6DOH	6-deoxyhexose pathways
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid

E.	*Escherichia*
Fe-EDTA	Iron-ethylenediaminetetraacetic acid
F6P	fructose-6-phosphate
Gap1	glyceraldehyde-3-phosphate dehydrogenase 1
Gap2	glyceraldehyde-3-phosphate dehydrogenase 2
Gdh	NDP-glucose dehydratase
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G3P	glyceraldehyde-3-phosphate
Gtt	NDP-glucose synthase
HPLC	high performance liquid chromatography
KR	ketoreductase
LB	Luria-Bertani
LC-MS	liquid chromatography-mass spectroscopy
MMT	methylmalonyl-CoA transcarboxylase
MutB	methylmalonyl-CoA mutase
mm	millimeter
mM	milimolar
MALDI-ToF-ToF-MS	matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
MetK	S-adenosyl-L-methionine synthetase

m/z	mass to charge ratio
mg	milligram
min	minute
ml	milliliter
NOESY	nuclear overhauser effect spectroscopy
nm	nanometer
NDYE	nitrate defined yeast extract
ORF	open reading frame
PEG	polyethylene glycol
PCC	propionyl-CoA carboxylase
PCR	polymerase chain reaction
PKS	polyketide synthase
Pgi	phosphoglucose isomerase
6PG	6-phosphogluconate
6PGL	6-phosphoglucolactone
Pgm	phosphoglucomutase
PPP	pentosephosphate pathway
THN	1, 3, 6, 8-tetrahydroxynaphthalene
Red	undecylprodigiosin
RT	retention time
RT-PCR	reverse transcription polymerase chain reaction

RNA ribonucleic acid

ROESY rotational-nuclear Overhauser Effect SpectroscopY

S. *Streptomyces*

SAM S-adenosyl- L-methionine

Sac. Erythraea *Saccharopolyspora erythraea*

SARP *Streptomyces* antibiotic regulatory protein

SDS sodium dodecyl sulfate

UV ultraviolet

µg microgram

µl microliter

X-gal 5-bromo-4-chloro-3-indolyl- -D-galactopyranoside

Zwf1 glucose-6-phosphate dehydrogenase 1

Zwf2 glucose-6-phosphate dehydrogenase 2

To

My Beloved Family

Chapter I

Introduction

1.1 Natural products and microbial secondary metabolites

Natural products are the best source of diversified chemotype for the discovery of novel therapeutics. About 60% of the antitumor and anti-infective agents that are commercially used are of natural products origin (Manly *et al*., 2002). Secondary metabolites are naturally produced substances that are not involved in normal growth, development or reproduction of an organism, and therefore are not essential to life. Microbial secondary metabolites are extremely diverse and important class of natural products, including clinically important antibiotics, antitumor compounds, immunosuppressants, antiviral, antiparasitic agents, pigments and herbicides along with agents utilized in agricultural, veterinary, and food industries (Gao *et al*., 2010). About 23,000 bioactive compounds produced by microorganisms have been reported, and approximately only 150 of them are being used routinely in pharmacology, agriculture or other fields. About 10,000 of these compounds are biosynthesized by actinomycetes, which represents 45% of known bioactive microbial metabolites. Among actinomycetes, *Streptomyces* species alone produce around 7600 compounds (Berdy, 2005).

For commercial use, large scale of drugs is obtained either by fermentative production, chemical synthesis or semisynthetic processes. However, natural compounds are preferred over synthetic medicine as synthetic compounds can cause side effects. The advantages of biosynthesis over chemical synthesis are environmental friendliness, chemical selectivity and molecular diversity. Since few decades, many efforts have been focused to replace chemical processes by biosynthesis and to improve existing microbial processes with

more efficient and genetically modified hosts. Moreover, complex compounds that cannot be synthesized chemically can be often biosynthesized by microorganisms (Manly *et al*., 2002).

For fermentation, it requires microbial strains capable of producing high titers of compound. Microbial fermentation has been the basis for production of large-scale of pharmaceutical products since the development of penicillin in the 1940s. However, wild-type strains isolated from nature are usually fastidious that produce only discrete amounts of a particular secondary metabolite, that make the isolation and production of such compounds for widespread clinical-use difficult.Therefore, isolation of the compound implies the need for production improvement to meet commercial requirements.

After years of intensive improvement trials using the traditional ''mutate-and-screen'', method, nowadays the titers of compounds made in industrial scale are very high. Today *Penicillium chrysogenum* produces over 70 g/L of penicillin which represents a 1000-fold increase. It used to produce only 60 mg/L of penicillin. Other examples of strain improvement are riboflavin production by *Ashbya gossypii* that has been improved 40,000 times and vitamin B12 production by *Pseudomonas denitrificans* that represents 100,000-fold increase (Demain, 2006).

Methods that are implied currently to increase the commercial production of pharmaceutically important drugs go from the classical random mutagenesis to the use of more rational methods. One of these methods is metabolic engineering where primary metabolic fluxes are redirected by genetic modifications through recombinant DNA technology, such that high production of secondary metabolite becomes possible (Adrio and

Demain, 2006). Moreover, the development of modern technologies such as DNA sequencing, transcription profiling, genomics, proteomics, metabolomics, transcriptomics and metabolite profiling has given many opportunities to engineer microorganisms for the high production of natural products (Bro and Nielsen, 2004).

1.2 Polyketides are secondary metabolites

The genetic capability of many soil microorganisms, particularly actinomycetes to synthesize antibiotics and other secondary metabolites is amazing. Polyketides, which are produced through the successive condensation of acyl-coenzyme A (acyl-CoA) precursors such as acetate, propionate, malonate or butyrate, by the action of multi-enzyme complex known as polyketide synthase, represents a large group of secondary metabolites with a broad range of structures and biological activities (Katz and Donadio, 1993). There are about 10,000 known polyketides and most of them have clinical significance. The polyketides encompass a variety of therapeutic drugs, including adriamycin (anticancer), erythromycin and tetracycline (antibiotics), mevacor and lovastatin (anticardiovascular disease) and sirolimus and tacrolimus (immunosuppressive drugs) (O'Hagan, 1991). Based on diversity in structure and function, polyketides are categorized into three classes.

Type I polyketides are a group of non-aromatized compounds synthesized by type I PKS system, a large multifunctional protein complex carrying all the active sites required for carbon chain assembly. Type I PKS system comprises large multifunctional polypeptides which are arranged in a modular fashion with each module being responsible for one round

of chain elongation and subsequent β-keto processing. In modular type I PKS, each active site is used only once during the polyketide biosynthesis. Some examples of type I polyketides include erythromycin, pikromycin etc (**Fig.1-1**). Iterative type I PKS possess only one multidomain protein, in which all the enzyme activities are covalently linked together. The single multifunctional protein is sufficient to catalyze multiple rounds of chain elongation and appropriate β-keto processing. In some iterative type I PKS, the one set of catalytic domains is able to vary the reduction level of β- keto groups during different extension cycles (Kennedy *et al*., 1999; Hendrickson *et al*., 1999). Some examples of iterative type I polyketides include 6-methylsalicyclic acid and lovastatin.

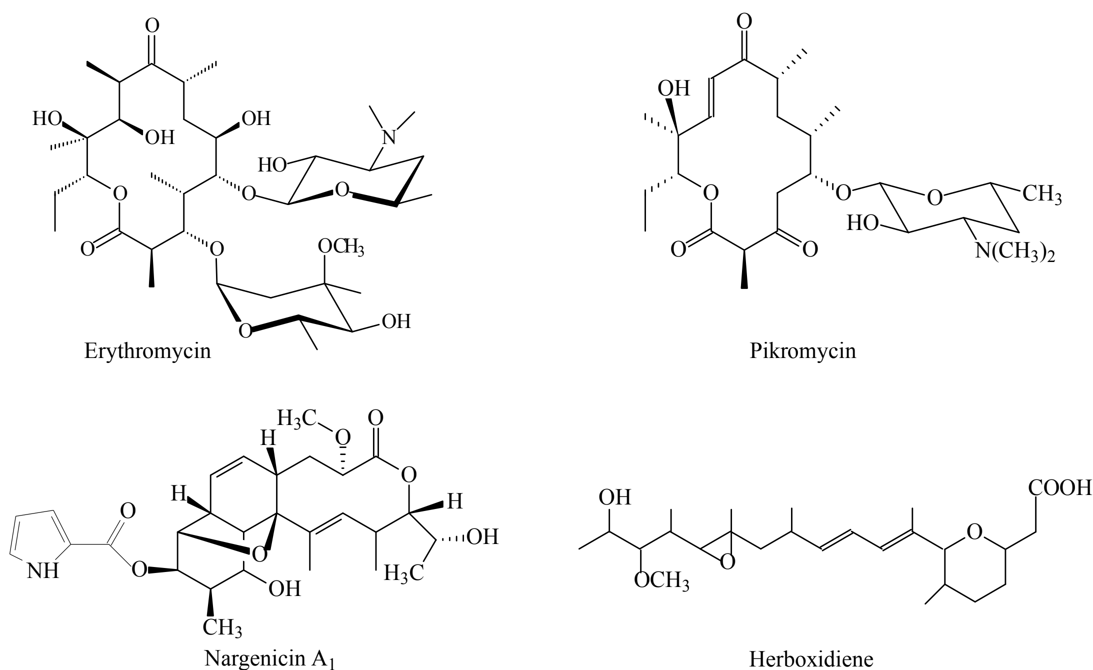


Figure 1-1. Examples of polyketide type I PKS compound.

Type II iterative polyketide compounds, also known as aromatic polyketides or anthracyclines, are produced mainly through the condensation of acetate groups, with the exception of the starter unit reactions, through several reaction steps catalyzed by type II PKS. Type II PKS comprises only one set of a heterodimeric ketosynthase (KSα-KSβ) and an acyl carrier protein (ACP) that operates to build an acyl chain with desired length which undergoes spontaneous or programmed folding by cyclase (CYC), ketoreductase (KR) and aromatase (ARO) to produce the aromatic structure. Some examples of type II polyketides include tetracenomycin C, doxorubicin and actinorhodin (**Fig.1-2**).

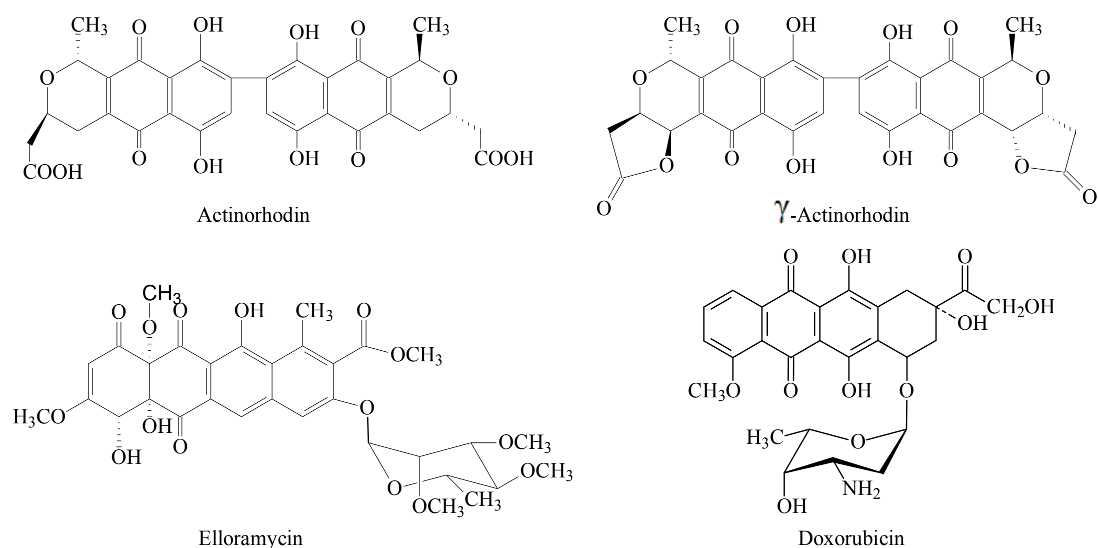


Figure 1-2. Examples of polyketide type II PKS compound.

Type III polyketides include chalcone synthase and stilbene synthase in plants and polyhydroxy phenols in bacteria. Homodimeric chalcone synthase and stilbene synthase are comparatively small proteins with a single polypeptide chain and are involved in the biosynthesis of tetraketide intermediates followed by cyclization to produce the final products. Type III PKS lacks traditional ACP and KS domains and use acyl-CoA substrates directly (**Fig.1-3**).

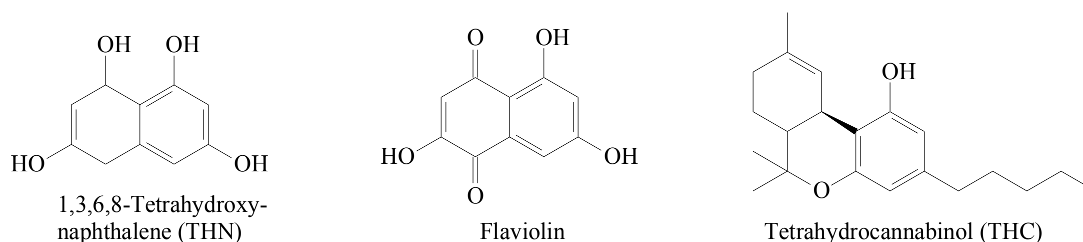


Figure 1-3. Examples of polyketide type III PKS compound.

1.3 Metabolic engineering: Different approaches used to improve production of secondary metabolites in actinomycetes

Metabolic engineering is an approach that involves targeted and purposeful alteration of metabolic pathways in an organism to understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly (Lessard, 1996). This multidisciplinary approach utilizes principles from chemical engineering, biochemistry, molecular biology as well as computational sciences. It involves the application of engineering principles of design and analysis to the metabolic pathways to achieve a particular goal such as to increase process productivity, biosynthetic precursors, or

to prolong metabolic capability by the addition of extrinsic activities for chemical production or degradation. The different genetic approaches that are used to improve production of secondary metabolites in actinomycetes include:

- Altering the distribution of metabolic flux of its different precursors
- Deregulating its biosynthetic pathway
- Increasing self-resistance and/or inducing resistance to several antibiotics
- Overexpressing biosynthetic genes of the metabolite
- Using global genetic approaches
- Using heterologous host or an industrially optimized strain for entire biosynthetic gene cluster expression

Some of the examples of these approaches are summarized in **Table 1-1** and schematized in **Fig. 1-4**.

Table 1-1. Increase secondary metabolites production by metabolic engineering

Compound	Strain	Engineering approach	Increase (fold)
Actinomycin	*S. antibioticus*	Ribosome engineering	5.25
Actinorhodin	*S. coelicolor*	Fatty acid precursors	6
		Up-regulation	2.6–40
		Ribosome engineering	1.6–180
C-1027	*S. globisporus*	Biosynthetic structural genes	2–4
Clavulanic acid	*S. clavuligerus*	Carbohydrate metabolism	3
		Biosynthetic structural genes	2.1–3.1
		Up-regulation	1.6–5
Desosaminyl tylactone	*S. venezuelae*	Heterologous expression,	17.1
		PKS deletion and up-regulation	
Doxorubicin	*S. peucetius*	Biosynthetic structural genes	4
		Up-regulation	2–4
Erythromycin	*A. erythreum*	Fatty acid precursors	2–4
	Sac. erythraea	Expression of heterologous genes	1.25–1.5
		Plasmid integration	2–2.5
Kanamycin	*S. kanamyceticus*	Self-resistance	3.5
Mithramycin	*S. argillaceus*	Up-regulation	2–16
Monensin B	*S. cinnamonensis*	Fatty acid precursors	1.76
Neomycin	*S. fradiae*	Self-resistance	6
Novobiocin	*S. coelicolor*	Heterologous expression	3
		up-regulation	
Pikromycin	*S. venezuelae*	Up-regulation	1.6–2.6
Spinosyn	*Sac. spinosa*	Carbohydrate metabolism	3
Undecylprodigiosin	*S. coelicolor*	Up-regulation	31
	S. lividans	Carbohydrate metabolism	4
		Down-regulation	11–12
		Ribosome engineering	1.9–2.9

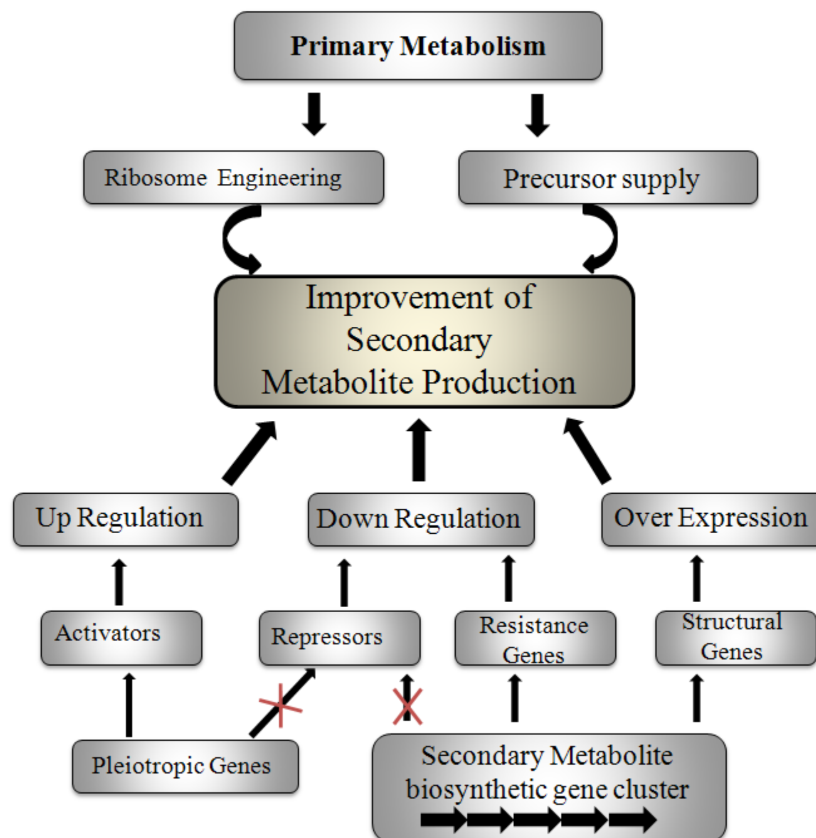


Figure 1-4. Schematic representation of different approaches used to improve secondary metabolite production.

1.3.1 Engineering polyketide precursors

It is well known that the availability of precursors is a key factor that determines the productivity of secondary metabolites. Primary metabolism supplies necessary precursors that are formed through the catabolism of different carbon substrates such as fatty acids,

monosaccharides or proteins. The genetic manipulation of key enzymes that regulate carbon flux through the metabolic network of central carbon metabolism leads to an increase in the availability of a particular precursor.

Embden-Meyerhof and pentose phosphate pathways (PPP) are interlinked to form the metabolic network in glucose catabolism (**Fig. 1-5**) where key enzymes in the individual pathways regulate the carbon flux among them. Studies have reported the genetic manipulation of the initial steps in the Embden-Meyerhof pathway and PPP for the enhanced production of clavulanic acid, actinorhodin and undecylprodigiosin. To increase the production of actinorhodin and undecylprodigiosin, PPP was engineered independently by removing *zwf1* and *zwf2*, coding for isoenzymes of glucose-6-phosphate dehydrogenase, and *devB* coding for a 6-phosphoglucolactonase in *S. lividans*. Deletion of these genes leads the channeling of precursors flux through the Embden-Meyerhof instead of PPP, which in turn leads to the increase of acetyl-CoA, which is the precursor of undecylprodigiosin and actinorhodin, thus increasing production of the antibiotics (**Fig. 1-5**) (Butler *et al*., 2002).

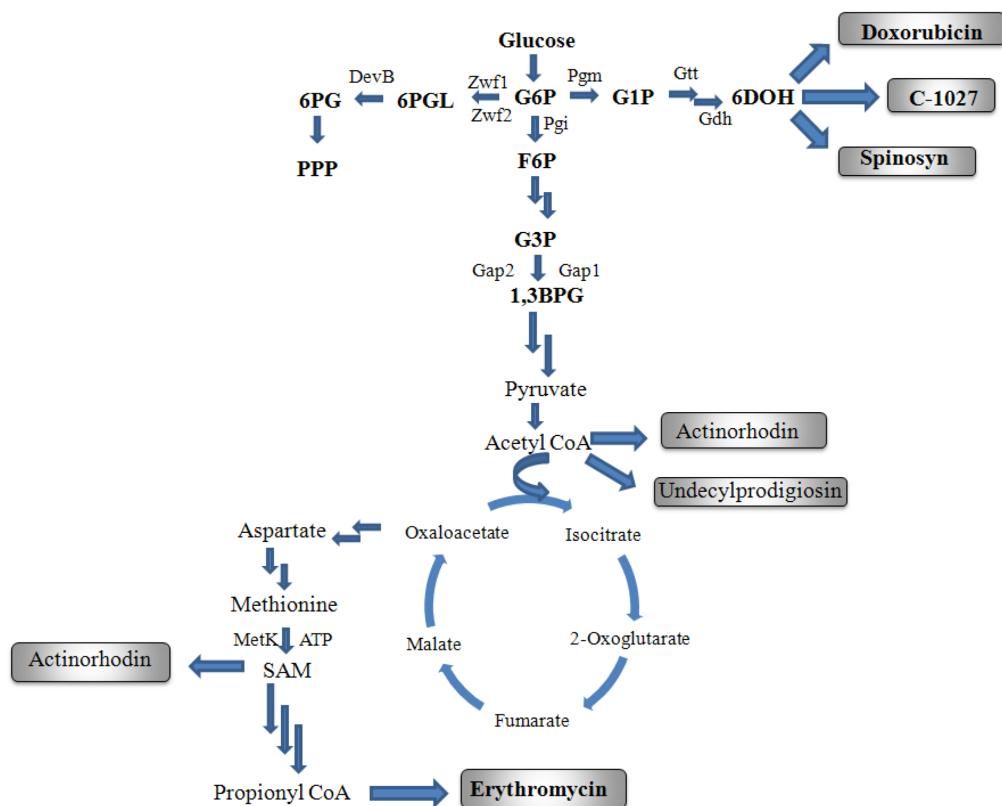


Figure 1-5. Primary metabolic pathways and engineered steps involved in the biosynthesis of several secondary metabolites produced by actinomycetes.

Enhanced production of several polyketides such as erythromycin, oligomycin, monensin B and actinorhodin in the native producer has been reported to be achieved by engineering the availability of coenzyme A (CoA) activated fatty acid precursors. Overproduction of erythromycin was achieved by inactivating the methylmalonyl-CoA mutase (MCM) gene *mutB* and cultivating producer strains, *Sac. erythraea* or *A. erythreum* in a carbohydrate-based medium. These experiments led to the conclusion that in a

carbohydrate-based medium, the MCM reaction acts like a drain on the methylmalonyl-CoA pool, but in an oil-based medium whereas the same reaction acts to fill the methylmalonyl-CoA pool (Reeves *et al*., 2004; Reeves *et al*., 2006, Reeves *et al*., 2007). Another example of enhanced production of a polyketide by modification of its precursor supplies is the increased production of actinorhodin by *S. coelicolor* in which overexpression of the genes *accA2, accB* and *accE*, coding for the different subunits of the enzyme acetyl-CoA carboxylase (ACC) was carried out in *S. coelicolor*. It was sufficient to increase carbon flux to malonyl-CoA, a precursor of actinorhodin together with acetyl-CoA that will result in a six-fold increase in actinorhodin production (Ryu *et al*., 2006).

Additional elements of the central carbon metabolism, such as the cofactor S-adenosyl-L-methionine (SAM), are also targets for metabolic engineering approaches. These cofactors take part in the biosynthesis of secondary metabolites as precursors. Overexpression of *metK* gene from *S. spectabilis,* coding for S-adenosyl-L-methionine synthetase that catalyzes the synthesis of SAM from ATP and L-methionine, resulted in enhanced production of actinorhodin in *S. lividans* and *S. coelicolor.* The enhanced production of actinorhodin in both *S. lividans* and *S. coelicolor* is the consequence of inducing the expression of pathway-specific transcriptional activator ActII-ORF4 (Kim *et al*., 2003; Okamoto *et al*., 2003). A similar effect was observed on the production of pikromycin in *S. venezuelae* by the expression of *metK1-sp* gene from *S. peucetius*. In this case, enhanced production of pikromycin was due to an increase in transcripts of the pathway-

specific transcriptional activator gene (*pikD*) and and a ketosynthase gene (*KS*) (Maharjan *et al*., 2008).

1.3.2 Engineering regulatory networks

Structural genes involved in the biosynthesis of secondary metabolites are usually grouped together in clusters in the genome including their pathway-specific regulatory genes. Pathway-specific regulators can be either activators or repressors having positive or negative effects respectively on the expression of genes. The number of pathway-specific positive regulatory genes ranges from one as in actinorhodin pathway (Fernandez-Moreno *et al*., 1991) to three as in daunorubicin pathway (Stutzman-Engwall *et al*., 1992; Otten *et al*., 1995; Otten *et al*., 2000). In addition, whereas some clusters contain both activators and repressors, such as tylosin biosynthetic pathway includes two activator and two repressor genes, (Stratigopoulos *et al*., 2004) other clusters do not contain regulatory genes such as in erythromycin pathway (Rawlings, 2001). Besides pathway-specific regulators, other regulatory genes that are generally located outside the biosynthetic gene cluster may exhibit a regulatory role on the production of multiple secondary metabolites, showing pleiotropic effects. The best-known example is the biosynthesis of several antibiotics, actinorhodin, calcium-dependent antibiotic, undecylprodigiosin and methylenomycin, by *S. coelicolor* under the control of specific regulators, *act*II-ORF4, *cdaR, redD* and *redZ*, which is also affected by several pleiotropic gene, *afs, abs* and *bld* (Huang *et al*., 2005).

In actinomycetes, the majority of the pathway-specific activators belong to the *Streptomyces* antibiotic regulatory protein (SARP) family which is characterized by the presence of a winged helix-turn-helix (HTH) motif towards the N-terminal region. Several studies have reported the increased production of different secondary metabolites by overexpression of SARP positive regulators, such as actinorhodin and undecylprodigiosin in *S. coelicolor* by *act*II-ORF4 and *redD* (Narva and Feitelson, 1990; Fernandez-Moreno *et al*., 1991) and undecylprodigiosin in *S. lividans* and *S. parvulus* by *redD* (Malpartida *et al*., 1990). In some cases, SARP family proteins functions as pleiotropic regulatory proteins that control the production of multiple secondary metabolites as well as morphological differentiation. For example, *afsR* gene from *S. coelicolor* was found to increase actinorhodin and undecylprodigiosin production by its overexpression in *S. lividans* (Horinouchi *et al*., 1983). In addition, overexpression of *afsR-p,* SARP pleiotropic activators homologue to AfsR, in *S. lividans, S. clavuligerus, S. griseus* and *S. venezuelae* leads to overproduction of actinorhodin, clavulanic acid, streptomycin and pikromycin, respectively (Parajuli *et al*., 2005; Maharjan *et al*., 2008).

Another strategy to overproduce secondary metabolites is the inactivation of pathway-specific or pleiotropic repressors as in the case of chromomycin, when a pathway-specific transcriptional repressor *cmmRII* is inactivated in *S. griseus subsp. griseus* chromomycin is overproduced (Menendez *et al*., 2007). When *actVB-orf10*, encoding a LysR-type transcriptional regulator gene was inactivated, actinorhodin was found to be overproduced in *S. lividans* (Martinez-Costa *et al*., 1999). Deletion of both *phoR* and *phoP*, a

well-known pleiotropic repressor system related with phosphate regulation of secondary metabolite production, has been recently reported to increase pimaricin production in *S. nataliensis* (Mendes *et al*., 2007) and actinorhodin and undecylprodigiosin production in *S. lividans* (Sola-Landa *et al*., 2003). Thus, it can be concluded that regulation of secondary metabolite pathways via overexpression of positive regulators and/or by inactivation of repressors is the promising approach for the improvement of their production (**Fig. 1-4**).

1.3.3 Engineering antibiotic resistance

Generally, antibiotic biosynthetic gene clusters include one or more resistance genes to overcome the toxic effects of their products. Resistance systems include enzymes for modifying the antibiotic target site, antibiotic inactivating enzymes and transport systems (Cundliffe, 1989; Mendez and Salas, 2001). In some cases these resistance systems are also involved in antibiotic biosynthesis (Olano *et al*., 1995; Menendez *et al*., 2007). For example, activation of antibiotic production was observed in *S. lividans* and *S. coelicolor* after mutation of *rpsL* gene, encoding the ribosomal protein S12, which confers resistance to streptomycin (Shima *et al*., 1996). An increase in actinorhodin production up to 180-fold in *S. coelicolor* has been recently reported depending on the culture medium used (Wang *et al*., 2008). Besides mutations conferring resistance to streptomycin, improvement of antibiotic production is also possible by mutations conferring resistance to other antibiotics such as gentamicin, paromomycin, geneticin, thiostrepton and lincomycin.

Thus, ribosome engineering is one of the rational approaches for antibiotic overproduction in different *Streptomyces* spp. by conferring resistance to several antibiotics mediated by mutant ribosomes (**Fig. 1-4**). Another approach for increasing production yields in native organisms is to increase self-resistance. This strategy has been used in several antibiotic overproducing organisms such as to enhance production of two aminoglycosides, kanamycin and neomycin, by introducing the gene encoding an aminoglycoside 60-*N*-acetyltransferase from *S. kanamyceticus* that confers resistance to aminoglycoside antibiotics in the producer strains (Crameri and Davies, 1986).

1.3.4 Engineering biosynthetic structural genes

Engineering of biosynthetic structural genes involved in the synthesis of secondary metabolites is carried out either to enhance production or to generate new compounds, usually through gene inactivation or deletion. The mutants thus generated can also be used as hosts for the heterologous expression of genes to generate new compounds. Several studies have been reported where enhanced production of secondary metabolites have been achieved by gene dose alteration, modification and heterologous expression of biosynthetic structural genes (**Fig. 1-4**). For improving production of antitumor drug, doxorubicin, different experiments have been carried out. One of the approaches was inactivation of *dnrX* and *dnrH* genes, involved in the conversion of daunorubicin and doxorubicin into baumycins in *S. peucetius* that resulted into 3- and 8.5-fold increased production of doxorubicin and daunorubicin respectively (Lomovskaya *et al*., 1998; Scotti and Hutchinson, 1996). Another

approach was disruption of *dnrU*, involved in the conversion of daunorubicin into 1,3-dihydrodaunorubicin, which resulted similar improvement of doxorubicin production. Similarly, the mutants generated by the disruption of several structural genes in the producer strain further improved the production of doxorubicin (Lomovskaya *et al*., 1999). Thus, deletion of some of the biosynthetic genes can raise the production of other secondary metabolite.

A new method for rapid enhancement of secondary metabolite production is genome shuffling. Six- to eight-fold increase in production of tylosin was obtained in *S. fradiae* by two rounds of genome shuffling over a population of classically improved strains (Zhang *et al*., 2002).

1.3.5 Expressing entire gene cluster heterologously

The production of secondary metabolites in some of the native producers is low and these strains are not amenable for metabolic engineering with available genetic tools. The consequence of these problems developed the use of new hosts which are previously engineered for the heterologous production of compounds. In such cases heterologous expression of entire gene cluster has been used as a genetic tool for the identification of a particular gene cluster and for improvement of the production yields. For example, Tetracenomycin C biosynthetic gene cluster was identified by its expression in *S. lividans*, which also resulted in the enhanced production of pigmented intermediates of the biosynthetic pathway (Motamedi and Hutchinson, 1987). Similarly, production of the

tylactone and its glycosylated derivative desosaminyl tylactone was achieved by the expression of entire tylosin PKS genes into a *S. venezuelae* strain where the pikromycin PKS genes were previously deleted to avoid competition for the acyl-CoA precursors (Jung *et al*., 2006). Furthermore, production of these compounds was increased by 2.7- and 17.1-fold by introducing an additional copy of *pikD*, pikromycin pathway-specific positive regulator gene (Jung *et al*., 2008). In the heterologous host, the pathways can be engineered more easily than in the wild-type strains. Metabolic engineering of novobiocin biosynthetic pathways in *S. coelicolor* M512 by introducing the regulatory gene *nov*G resulted in three-fold overproduction of novobiocin (Eustaquio *et al*., 2005). Another example is the heterologous expression of *matB* and *matC* genes from *Rhizobium trifolii* by which four-fold enhanced production of 6-deoxy-erythronolide B (6-dEB) was achieved in *S. coelicolor* (Lombo *et al*., 2001).

Since the genetic tools for engineering *E. coli* strains are highly developed and it requires less fermentation time, heterologous expression of gene clusters in *E. coli* to overproduce desired compounds would be more appropriate. Many efforts have been made for the production of erythromycin in *E. coli* such as expression of phosphopantetheinyl transferase gene, *sfp* from *Bacillus subtilis* and expression of PCC genes (*pccA* and *pccB)* from *S. coelicolor*. The resultant *E. coli* strain produced equivalent amount of 6-dEB as produced by *Sac. erythraea* industrial strain (Pfeifer *et al*., 2001). Further expression of *metK* from *S. spectabilis* led to a two-fold improvement in 6-dEB production (Wang *et al*., 2007). Besides polyketides, other secondary metabolites such as non-ribosomal peptides are

also produced in *E. coli* expressing the entire gene cluster along with *sfp* gene for post-translational modification of the NRPS (Watanabe *et al*., 2006).

The optimized host for maximum production of natural products will likely to have all of the metabolic engineering strategies in the form of an ‘‘integrated system’’ that optimizes heterologous and native metabolism towards the goal of optimized final polyketide production (Boghigian *et al*., 2008) (**Fig. 1-6**).

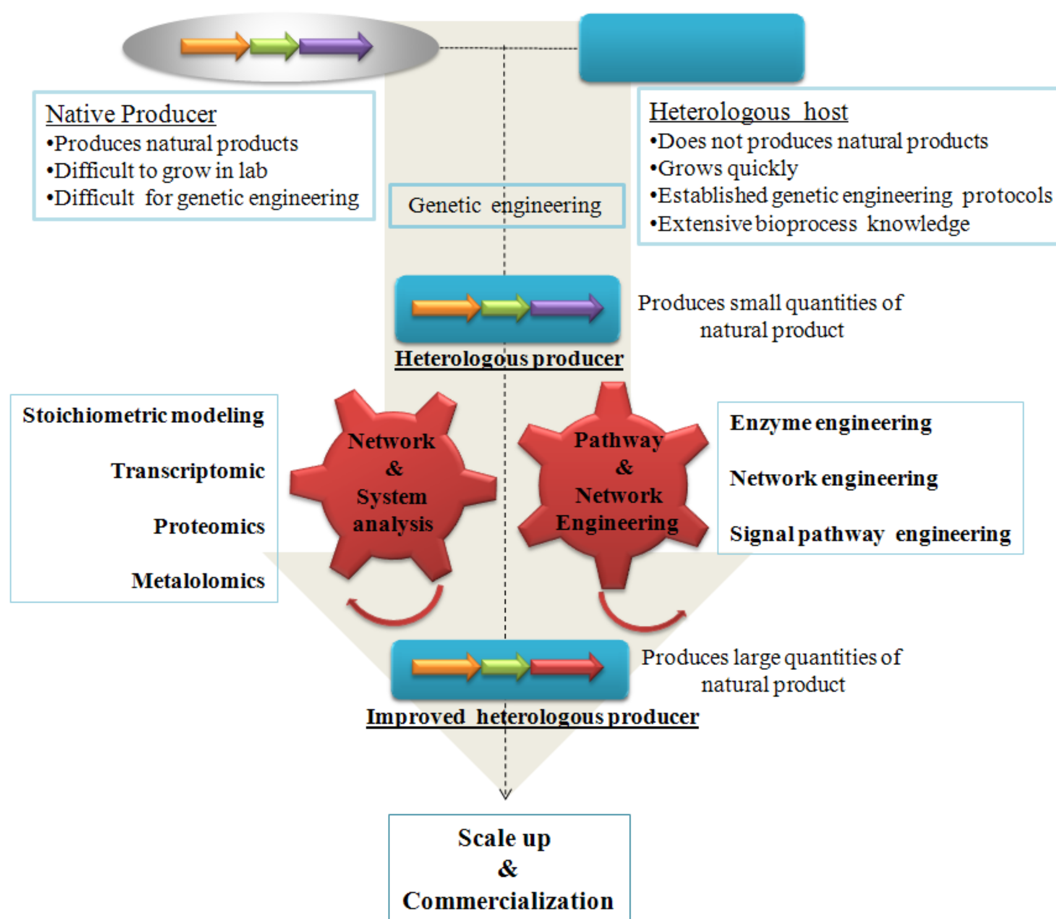


Figure 1-6. Schematic representation of optimized "integrated system'' for improving heterologous natural product biosynthesis.

1.4 Objective of the study

Nature is provided with a huge number of microorganisms capable of producing different secondary metabolites with important biological functions including antibiotic, anticancer, immunosuppressant, and anti-cholesterol activities. Polyketides are the most important group of natural products exhibiting an enormous range of functional and structural diversity, 45% of which are produced by actinomycetes. To access these diversified therapeutically important compounds for biomedical applications, these actinomycetes have been engineered to produce higher levels and/or novel compounds.

The development of recombinant DNA technology has provided us useful tools for improving secondary metabolites production by means of genetic manipulation of the organism. In this study, our strategy was to access metabolic engineering approaches via, recombinant DNA technology in order to boost the production of the bioactive compounds from native producer organisms and heterologous hosts.

Chapter II

Materials and methods

2.1 Bacterial strains, vectors, recombinant plasmids and growth condition

All bacterial strains used in this study are listed in **Table 2-1**. *E. coli* XL1 Blue MRF' (Stratagene) was used for DNA amplification. *E. coli* ET 12567 was used to propagate non-methylated DNA. *E. coli* strains were grown at 37ºC in Luria-Bertani (LB) media in both liquid broth and agar plates supplemented with the appropriate antibiotics such as ampicillin (100 µg/ml), kanamycin (100 µg/ml), tetracycline (25 µg/ml), chloramphenicol (100 µg/ml) and apramycin (100 µg/ml) when necessary. All vectors, cosmids and plasmids used in this study are listed in **Table 2-2**. pGEM-T Easy vector (Promega, USA) and pGEM-7Z (+) vector were used as the cloning vector. The plasmids, pIBR25 and pSET152, were used as the expression and integration vectors respectively for *Streptomyces* strains. SuperCos I cosmid was used for construction of library.

All *Streptomyces* strains were grown at 28ºC in R2YE media in order to prepare the protoplasts and isolate plasmid DNA. Protoplast transformation was carried out according to the standard protocol (Kieser *et al*., 2000). Recombinant plasmids were transformed into protoplasts of *S. peucetius* ATCC 27952, *S. lividans* TK24, and *S. venezuelae* ATCC 15439, *S. venezuelae* YJ028 and *S. chromofuscus* according to the standard protocol. Thiostrepton (12.5 µg/ml for *S. peucetius* and 50 µg/ml for *S. lividans* TK24) and apramycin (500 µg/ml for *S. venezuelae* and 100 µg/ml for *S. chromofuscus*) were used to select recombinant strains. SCM liquid media (Semiselective Media containing 1.5% soluble starch, 2% Soytone, 0.01% $CaCl_2$, 0.15% yeast extract, and 1% MOPS) was used to isolate pikromycin and flaviolin from *S. venezuelae* strains. NDYE medium (11.2% maltose, 0.7% yeast extract,

21.4% $NaNO_3$, 1.15% K_2HPO_4, 23.8% HEPES, 0.6% $MgSO_4 \cdot 6H_2O$, 4% NaOH), supplemented with 1 ml of inorganic solution (1 mg of ferrous sulfate, 1 mg of magnesium chloride, and 1 mg of zinc sulfate per 1,000ml distilled water), was used for the production of doxorubicin from *S. peucetius* and its recombinant strains. YEME medium (1% glucose, 0.3% malt extract, 0.5% peptone, 34% sucrose, 0.3% yeast extract, and 10 mM magnesium chloride) was used as production media for actinorhodin from *S. lividans* TK24 and its recombinant strains. *S. chromofuscus* ATCC 49982 (*Streptomyces* sp. A 7847), *S. chromofuscus*-SET152, *S. chromofuscus*-ACC152, *S. chromofuscus*-ASA152, *S. chromofuscus*-PCC152 and *S. chromofuscus*-PSA152 were grown in ISP2 agar plates and in ISP2 liquid media. Production media comprising 3.5% corn starch, 0.8% proflo, 0.1% $MgSO_4$, 0.2% KH_2PO_4, 0.2% KNO_3, 0.05% NaCl, 0.015% $CaCO_3$, 0.001% $ZnSO_4$ and 0.018% Fe-EDTA was used as production media for herboxidiene.

Reverse transcription polymerase chain reaction (RT-PCR) was carried out using APM medium (6% glucose, 2% malt extract, 1.5% MOPS, 0.2% sodium chloride, and 0.8% yeast extract).

For extraction of intracellular acetyl-CoA and malonyl-CoA, strains were grown on SPA agar plates (dry nutrient agar based on Sprat hydrolysate containing 0.1% yeast extract, 0.1% beef extract, 0.2% tryptose, 1% glucose, 1.5% agar, and trace amount of $FeSO_4$) at 30ºC in order to isolate an individual colony, which was subsequently used to inoculate 50 ml of SCM media (Semiselective Media containing 1.5% soluble starch, 2% Soytone, 0.01% $CaCl_2$, 0.15% yeast extract, and 1% MOPS) in a baffled Erlenmeyer flask.

Table 2-1. Bacterial strains used in this study

Strains	Description	Source
E.coli XL1 Blue	General cloning host	Strategene
E.coli ET12567	Demethylation host	Strategene
E.coli ET12567/ETPUZ	Demethylation host	Strategene
S. venezuelae ATCC 15439	Pikromycin producer wild strain	ATCC
S. venezuelae/pSET152	*S. venezuelae* harboring pSET152	This study
S. venezuelae/pIBR25	*S. venezuelae* harboring pIBR25	This study
*S. venezuelae/*pSIBR	*S. venezuelae* harboring pSIBR	This study
S. venezuelae/pGIBR	*S. venezuelae* harboring pGIBR	This study
S. venezuelae/pASV152	*S. venezuelae* harboring pASV152	This study
S. peucetius ATCC 27952	Doxorubicin producer wild strain	ATCC
S. peucetius/pIBR25	*S. peucetius* harboring pIBR25	This study
S. peucetius/pASV25	*S. peucetius* harboring pASV25	This study
S. lividans TK24	Actinorhodin producer	This study
S. lividans TK24/pIBR25	*S. lividans* TK24 harboring pIBR25	This study
S. lividans TK24/pASV25	*S. lividans* TK24 harboring pASV25	This study
S. venezuelae YJ028	Pik-PKS and des-genes deleted mutant of *S. venezuelae*	Jung *et al*., 2007
S. venezuelae 28-SET152	*S. venezuelae* YJ028 harboring pSET152	This study
S. venezuelae 28-ACC152	*S. venezuelae* YJ028 harboring pACC152	This study
S. venezuelae 28-ASA152	*S. venezuelae* YJ028 harboring pASA152	This study
S. venezuelae 28-ACC152/GG1	*S. venezuelae* 28-ACC152 harboring pGG1	This study
S. venezuelae 28-ASA152/GG1	*S. venezuelae* 28-ASA152 harboring pGG1	This study
S. venezuelae 28-GG1	*S. venezuelae* YJ028 harboring pGG1	This study
S. chromofuscus ATCC 49982	Herboxidiene producer wild strain	ATCC
S. chromofuscus-SET152	*S. chromofuscus* harboring pSET152	This study
S. chromofuscus-ACC152	*S. chromofuscus* harboring pACC152	This study
S. chromofuscus-ASA152	*S. chromofuscus* harboring pASA152	This study
S. chromofuscus-PCC152	*S. chromofuscus* harboring pPCC152	This study
S. chromofuscus-PSA152	*S. chromofuscus* harboring pPSA152	This study

Table 2-2. Vectors and recombinant plasmids used in this study

Plasmids	Description	Source
pGEM-T Easy	*E. coli* cloning vector, *amp*^{r}	Promega
pGEM-7Z (+)	ColE1 pMB1 ori, bla (*amp*^{r}), LacZ-α, subcloning vector	Promega USA
pIBR25	*Streptomyces* expression vector,with *ermE** promoter and *tsr*^{r}	Sthapit *et al*., 2004
pSET152 ermE*	*Streptomyces* integration vector with *ermE** promoter and *apr*^{r}	Malla *et. al.* (unpublished)
pGIBR	pIBR25 based recombinant plasmid harboring *afsR-p*	Parajuli *et al*., 2005
pSIBR	pIBR25 based recombinant plasmid harboring *metK1-sp*	This study
SuperCosI	7.9 kb cosmid vector	Stratagene
pASV25	pIBR25 based recombinant plasmid harboring *afsR-sv*	This study
pASV152	pSET152 based recombinant plasmid harboring *afsR-sv*	This study
pACC152	pSET152 based recombinant plasmid harboring acetyl-CoA carboxylase *(accA2, accB* and *accE*)	This study
pASA152	pSET152 based recombinant plasmid harboring acetyl-CoA carboxylase, *metk1-sp* and *afsR-sp*	This study
pGG1	pIBR25 based recombinant plasmid harboring *sp-rppA*	Ghimire *et al*., 2008
pMetK25	pIBR25 based recombinant plasmid harboring *metK1-sp*	This study
pPCC152	pSET152 based recombinant plasmid harboring propionyl-CoA carboxylase (*prpE, accA1, pccBE*)	This study
pPSA152	pSET152 based recombinant plasmid harboring propionyl-CoA carboxylase, *metK1-sp* and *afsR-sp*	This study
pNV18	*Nocardia-E.coli* shuttle vector	Chiba *et al*., 2007
pNVmetK18	pNV18 harboring *metK1-sp*	This study
pNVacc	pNV18 harboring acetyl-CoA carboxylase	This study

2.2 Chemicals and reagents

All restriction enzymes, DNA ligase, LA Taq polymerase, PCR premix and calf intestinal alkaline phosphatase used in this study were purchased from Takara Company (Japan), Novagen (Darmstadt, Germany) and Genotech Co. Ltd. (Daejeon, South Korea).The antibiotics ampicillin, apramycin, kanamycin, tetracycline, chloramphenicol and neomycin, X-gal and IPTG were purchased from Sigma Aldrich Chemical Company (St. Louis, Missouri, USA). Hybond-N nylon membrane and DIG high prime DNA labeling and detection starter kit used in Southern blot were purchased from Amersham (Braunschweig, Germany) and Roche Molecular Biochemicals (Mannheim, Germany) respectively. Gigapack III XL packaging material used in construction of library was purchased from Stratagene Company (U.S.A.). An RNeasy Mini kit used for RNA isolation and purification was purchased from Qiagen (Hilden, Germany). All high performance liquid chromatography (HPLC) grade organic solvents used for HPLC analyses were purchased from Mallinckrodt Baker (Phillipsburg, USA) and all other organic solvents used for compound isolation were purchased from Daejung Chemicals and Metals Co. Ltd. (Daejung, Korea). All media used to culture *E. coli* and *Streptomyces* strains were purchased from Becton, Dickinson and Company (Sparks, USA). All other chemicals and reagents were high grade products obtained from commercially available sources.

2.3 Polymerase chain reaction

Oligonucleotide primers used in this study for amplification of desired gene are listed in **Table 2-3**. PCR premix (Genotech, Korea) was used for preliminary optimizing the PCR conditions. The PCR reaction was performed in TaKaRa PCR Thermal Cycler Dice (Takara, Japan) with LA Taq polymerase according to the manufacturer's instructions. The total volume of 20 µl PCR reaction mixture contained 20 pM primers, 0.125 mM dNTPs, 10% DMSO, 2 units of LA Taq polymerase, 2.5 mM $MgCl_2$, appropriate amount of template, PCR buffer and double distilled water. The PCR reaction conditions were as follows; Denaturation at 94ºC for 7 min, 30 cycles of denaturation at 97ºC for 1 min, annealing at 60–70ºC for 1 min, and polymerization at 72ºC for 1 min.

Table 2-3. Primers used in this study.

Gene	Sequences(5'−3')	Restriction sites
metK1-F	TTGGGGATCCATGTCCCGTC	*Bam*HI
metK1-R	CCGCAAGCTTCTGTGCACTCA	*Hin*dIII
metK1-NF	GGCTCTAGAGTCCTTTGGAGTTCCATG	*Xba*I
metK1-NR	CACTGCAGCATGCTGCTGTGCACTCA	*Pst*I
afsR-pF	TCCGGAATTCCCGGCAGGGGGC	*Eco*RI
afsR-pR	CGAAGCTTCGGACCGAGCACGA	*Hin*dIII
metK1-RTF	GCAAGACCCAGGTCACCATCGAGTACC	
metK1-RTR	GTCGAAGACCTGGGTGATGGCCTTCTC	
afsr-pRTF	TACCGGTACCACGACCTTGTGCGTCT	
afsr-pRTR	TGTTCGTCGGCCTCGTCGTAACGGC	
pikD-RTF	CATCTCAGGGCCGTTCTTGACGCATCC	
pikD-RTR	CGTAGTAGTGGGCGAGTAACTGGCGTAC	
16S rRNA-RTF	CCTTCGGGTTGTAAACCTCTTTCAGCA	
16S rRNA-RTR	CAACACCTAGTTCCCAACGTTTACGGC	
KS-RTF	CGAACGGTTTCGCGGAGCAGACTCATG	
KS-RTR	GAAGTCGTGGTGCACCACGGTCACATG	
CPF	GCSGGSATCGGSGGSGTSGGSAAGACSA	
CPR	CTCGTCSGCSAGCTTSGCSGCSAGSACSGA	
afsR-svF	AAAGAATTCGTACGGGCAGGGGGAGCT	*Eco*RI
afsR-svR	ATAAAGCTTGAACGCCCAGGCCGGGAC	*Hin*dIII
metK1-spF	GGCTCTAGAGTCCTTTGGAGTTCCATG	*Xba*I
metK1-spR	CAGAATTCCATGCTGCTGTGCACTCA	*Eco*RI
afsR-spF	TCCGGAATTCCCGGCAGGGGGC	*Eco*RI
afsR-spR	GGAAGCTTTCTAGACGGACCGAGCACGA	*Hin*dIII

2.4 Construction of genomic library and sequence analysis

Genomic DNA was isolated from *S. venezuelae* by lysozyme treatment and phenol-chloroform extraction (Kieser *et al*., 2000). A cosmid library of *S. venezuelae* was constructed by partial *Sau*3AI digestion of chromosomal DNA, and ligation into the *Xba*I-*Bam*HI of SuperCosI. Packaging was performed with Gigapack III XL (Stratagene, U.S.A.) and transduced into *E. coli* XL1-Blue MRF'. Common primers, CPF and CPR, were designed where S may be replaced by G or C based on the conserved residues from *afsR-sp* (*S. peucetius*), *afsR-g* (*S. griseus*) and *afsR* (*S. coelicolor*). The 600 bp DNA fragment was amplified from *S. venezuelae* using those primers. The PCR products were ligated into the pGEM-T easy vector, sequenced to confirm that no mutation had occurred during PCR amplification and used as probes for library screening. For hybridization experiments, the probes were labeled with digoxigenin high prime DNA labeling and detection start kit II (Roche Molecular Biochemicals, Mannheim, Germany).

E. coli transfectants from library stock were diluted and plated onto Luria broth plates containing ampicillin (100 μg/ml). After overnight growth at 37°C, colonies were transferred to nylon membrane filters for in situ colony hybridization analysis according to published methods and screening was done by using a labeled probe that was generated using the DIG DNA labeling and detection kit. For second colony hybridization, selected clones from first hybridization were replica plated onto Luria Bertani agar plates containing ampicillin (100 μg/ml). Selected cosmids from second colony hybridization were digested with *Bam*HI and southern blot analysis was performed on Hybond-N nylon membranes with

digoxigenin-labeled probes by using the DIG high prime DNA labeling and detection starter kit II (Sambrook *et al*., 1989).

2.5 DNA sequencing and computer-assisted sequence analysis

Restriction fragments of approximately 300 to 5,000 bp obtained from the cosmid was subcloned into the pGEMT-Easy vector (Promega, U.S.A.). Sequencing was performed according to the dideoxynucleotide chain termination method on an automatic sequencer. The DNASIS software package (version 2.1, 1995; Hitachi Software Engineering, San Bruno, CA, U.S.A.) and BLAST (NCBI) were used for sequence analysis and homology search in the GenBank database respectively. The amino acid sequence of AfsR-sv was aligned with its homologs using the ClustalX program, and the conserved domain was analyzed using the Pfam 22.0 (Pfam Consortium) and NCBI database. The nucleotide sequence of *afsR-sv* reported in this paper has been deposited in the NCBI nucleotide sequence database under Accession No. EF612792.

2.6 Construction of expression and integration plasmids

The expression vector, pIBR25, under the control of the *ermE** promoter, which leads to the expression of DNA in *Streptomyces* species, was used for cloning (Sthapit *et al*., 2004). The *metK1-sp* was amplified by PCR of genomic DNA from *S. peucetius*, using metK1-F and metK1-R primers. The PCR product (1,209 bp) of the *metK1-sp* was cloned into the *Bam*HI-*Hin*dIII of pIBR25 to produce the recombinant plasmid pSIBR. Similarly,

afsR-sp from *S. peucetius* was amplified using afsR-pF and afsR-pR primers. The PCR product (2,946 bp) was cloned into the *Eco*RI-*Hin*dIII of pIBR25 to produce the recombinant plasmid pGIBR (Parajuli *et al*., 2005).

Two oligonucleotides, afsR-svF and afsR-svR were synthesized with *Eco*RI-*Hin*dIII, respectively. These were then used to amplify the *afsR-sv* from the genomic DNA of *S. venezuelae*. The PCR product (3,171 bp) obtained was purified and cloned into the *Eco*RI-*Hin*dIII of pIBR25 under the control of the *ermE** promoter in order to generate the expression recombinant plasmid pASV25. Similarly, the purified PCR product was subcloned into pGEM-7Z (+) and then cloned into the *Xba*I-*Bam*HI of the integration vector pSET152 in order to generate the integration recombinant plasmid pASV152. The SAM synthetase gene (*metK1-sp)* and *afsR-sp* were amplified from genomic DNA of *S. peucetius* ATCC 27952 using primer pairs, metK1-spF/metK1-spR and afsR-spF/afsR-spR respectively. The PCR product of *metK1-sp* (1,294 bp) was cloned into the *Xba*I-*Eco*RI of pIBR25 to obtain pMetK25. Similarly, the PCR product of *afsR-sp* (2,949 bp) was cloned into the *Eco*RI-*Hin*dIII of recombinant pMetK25 to yield pMA25. The *Xba*I-*Xba*I fragment, comprising *metk1-sp* and *afsR-sp*, was purified from pMA25 and cloned into same site of pACC152 and pPCC152 to obtain pASA152 and pPSA152 respectively. The recombinant pGG1 harboring *sp-rppA* from *S. peucetius* was used for flaviolin production (Ghimire *et al*., 2008). In every case, the PCR products were cloned into the pGEM-T vector and sequenced separately prior to cloning into the pSET152 and pIBR25 to confirm that no mutation had occurred during PCR amplification.

To carry out the experiments in *Nocardia* sp. CS682, the *Nocardia*-*E. coli* shuttle vector, pNV18, which leads to the expression of gene in *Nocardia* species, was used as expression vector and pMBE101 was used as subcloning vector. For the heterologous expression of *metK1-sp* (1,294 bp), it was amplified from the genome of *S. peucetius* ATCC 27952 using the primers metK1-NF/metK1-NR and cloned into *Xba*I-*Pst*I of pNV18 vector to generate recombinant plasmid pNVmetK18. For the expression of ACC, since pNV18 vector doesnot contain *Pac*I restriction site, *Xba*I-*Pac*I fragment of pACC152, comprising ACC genes, was first cloned into subcloning vector, pMBE101 resulting pMBEACC. Then *Xba*I-*Eco*RI fragment was taken out from pMBEACC and ligated into same sites of pNV18 generating pNVACC18, which was used as the recombinant plasmid for the expression of ACC in *Nocardia* sp. CS682.

2.7 Feeding experiment

For feeding experiment, L-proline solution was made by dissolving in sterilized double-distilled water and added in three portions (40%, 30% and 30%) through a disposable sterile filtration unit to each of 80ml culture broth of *Nocardia* sp. wild type and *Nocardia* sp. ACC18 after 24, 48 and 72 h. The total dose of L-proline was 3.0 g/L in each case.

2.8 Isolation and analysis of compounds

2.8.1 Isolation of pikromycin

Recombinants pSIBR and pGIBR were transformed into wild type *S. venezuelae* to obtain *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR respectively. Similarly, the plasmids pASV152 and pSET152 were integrated into the genomic DNA of *S. venezuelae* to generate *S. venezuelae*/pASV152 and *S. venezuelae*/pSET152 respectively. These transformations were done by following the PEG-mediated protoplast transformation method (Kieser *et al*., 2000).

S. venezuelae, *S. venezuelae*/pSIBR, *S. venezuelae*/pGIBR, *S. venezuelae*/pSET152, and *S. venezuelae*/pASV152 were grown in 50 ml of liquid SCM medium for 60 h at 28ºC. The culture broth of each strain was centrifuged for 15 min at 6,000 × g to remove cell pellets. The supernatants were extracted with 2 volumes of ethyl acetate, and the extract was dried under reduced pressure using a rotary evaporator and reconstituted by 1.5 ml of methanol. A 15 µl aliquot of the extract obtained was then analyzed on HPLC using a reverse-phase C-18 column (Mytstil RP-18, 4.6 × 250 mm × 5 µm) with 80% acetonitrile in 5 mM ammonium acetate buffer containing 0.05% acetic acid, adjusted to pH 8 with ammonium hydroxide. Detection was carried out with a UV absorbance detector, monitoring peaks at 220 nm for 66 min at a flow rate of 1 ml/min. The major peak corresponding to pikromycin was confirmed by liquid chromatography/mass spectrometry (LC/MS) analysis. The antibacterial activity of compounds from *S. venezuelae*, *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR was assayed against *Bacillus subtilis* ATCC 23857 (Lee *et al*., 2006).

2.8.2 Isolation of doxorubicin

The expression plasmids pIBR25 and pASV25 were transformed into *S. peucetius* generating *S. peucetius*/pIBR25 and *S. peucetius*/pASV25 respectively. *S. peucetius*, *S. peucetius*/pIBR25, and *S. peucetius*/pASV25 were grown in 50 ml of liquid NDYE medium for 84 h at 28°C. 50 ml of culture broth of each strain was centrifuged for 15 min at 6,000 × g to remove cell pellets. The supernatant was extracted with 2 volumes of $CHCl_3$:CH_3OH (9:1). The extract was dried under reduced pressure using a rotary evaporator and reconstituted with 1.5 ml of methanol. A 15 µl aliquot of the extract obtained was analyzed by HPLC using a reverse-phase C-18 column with 100% methanol (solvent B) and distilled water (solvent A, pH 2.34) for 71 min, with a flow rate of 1 ml/min. Detection was carried out using a UV absorbance detector, monitoring peaks at 254 nm. Authentic doxorubicin was used as a reference.

2.8.3 Isolation of actinorhodin

Similarly, the expression plasmids pIBR25 and pASV25 were also transformed into *S. lividans* TK24 to generate *S. lividans* TK24/pIBR25 and *S. lividans* TK24/pASV25 respectively.

S. lividans TK24, *S. lividans* TK24/pIBR25, and *S. lividans* TK24/pASV25 were cultured in 50 ml of liquid YEME medium for 9 days at 28°C. After centrifugation for 15 min at 6,000 × g to remove cell pellets, the pH of the supernatants was adjusted at 2.0 with 1 N HCl and extracted with chloroform. The amount of the blue-colored antibiotic,

actinorhodin was determined by measuring the optical density of the extracts at 633 nm using a UV spectrophotometer (Shimadzu, Japan) (Bystrykh *et al*., 1996). In every case, *Streptomyces* strains transformed with only vector were used as a control, and the productions were averaged from four separate cultivations and extractions.

2.8.4 Isolation of flaviolin

The integrative plasmids pSET152, pACC152 and pASA152 were integrated into the genomic DNA of *S. venezuelae* YJ028 to generate *S. venezuelae* 28-SET152, *S. venezuelae* 28-ACC152, and *S. venezuelae* 28-ASA152 respectively following the standard protoplast transformation protocol (Kieser *et al*., 2000). Transformants in each case were selected with apramycin (500 µg/ml). Integration of pSET152, pACC152 and pASA152 into chromosomal DNA of *S. venezuelae* YJ028 was confirmed by PCR of the apramycin resistance gene from total DNA isolated from *S. venezuelae* 28-SET152, *S. venezuelae* 28-ACC152 and *S. venezuelae* 28-ASA152. A plasmid pGG1 harboring sp-rppA was transformed into *S. venezuelae* 28-ACC152 and *S. venezuelae* 28-ASA152 resulting in *S. venezuelae* 28-ACC152/GG1 and *S. venezuelae* 28-ASA152/GG1 respectively. *S. venezuelae* YJ028 was also transformed with pGG1 to generate *S. venezuelae* 28-GG1 for comparative study. Transformants in each case were selected with apramycin (500 µg/ml) and thiostrepton (500 µg/ml). Transformation of pGG1 was confirmed by isolation and restriction enzyme digestion of plasmid from each strain.

S. venezuelae YJ028 and all its transformants were cultured in 50 ml of R2YE media containing appropriate antibiotics, apramycin (500 μg/ml) and thiostrepton (500 μg/ml), at 28ºC for 48 h. 500 μl of seed culture of each strain was inoculated into 80 ml SCM production media at 28ºC for 96 h. The culture broth of each strain was centrifuged for 15 min at 6,000 × g to remove cells, and the supernatants were acidified to pH 2 with H_2SO_4 and extracted twice with 2 volume of ethyl acetate. The ethyl acetate was removed using a rotary evaporator and the residue was dissolved in 1 ml methanol. The resultant products were analyzed by HPLC using a reverse-phase C-18 column (Mytstil RP-18), (Buffer A: 0.05% TFA in water, Buffer B: acetonitrile, solvent gradient from 10% B at 0 min to 30% B in 5 min, from 30% B to 60% B in 25 min and to 90% B in 40 min) at 0.5 ml/min with detection at 254 nm, LC-ESI/MS and GC/MS. The amount of flaviolin was determined by spectrophotometry on the basis of the molecular extinction coefficient (4.12) at 262 nm. The average production of flaviolin was determined from three separate cultivations and extractions.

2.8.5 Isolation of herboxidiene

500 μl of seed culture of *S. chromofuscus* ATCC 49982, grown in ISP2 media, was inoculated into 80 ml of production media (3.5% corn starch, 0.8% proflo, 0.1% $MgSO_4$, 0.2% KH_2PO_4, 0.2% KNO_3, 0.05% NaCl, 0.015% $CaCO_3$, 0.001% $ZnSO_4$, 0.018% Fe-EDTA) and was incubated at 28ºC for 7 days for extraction of herboxidiene. The culture broth was centrifuged for 15 min at 6,000 × g to remove cell pellets and the supernatant was

extracted with a double volume of ethylacetate. The solvent was evaporated and then concentrated with 1.0 ml of methanol. A 15 μl aliquot of the extract was analyzed on HPLC analysis using a reverse-phase C-18 column (Mytstil RP-18) with Buffer A: 0.05% TFA in water and Buffer B: acetonitrile, at 1.0 ml/min. Detection was carried out with a UV absorbance detector, monitoring at 236 nm. The major peak corresponding to herboxidiene was confirmed by liquid chromatography/mass spectrometry (LC/MS) analysis.

2.8.6 Isolation of blue and red compounds

The integrative plasmids pSET152, pACC152, pASA152, pPCC152 and pPSA152 were transformed separately into *S. chromofuscus* ATCC 49982 to generate *S. chromofuscus*-SET152, *S. chromofuscus*-ACC152, *S. chromofuscus*-ASA152, *S. chromofuscus*-PCC152 and *S. chromofuscus*-PSA152 respectively, following the standard protoplast transformation protocol (Kieser *et al*., 2000). Transformants in each case were selected with apramycin (50 μg/ml). Integration of pSET152, pACC152 and pASA152 into chromosomal DNA of *S. venezuelae* YJ028 was confirmed by PCR of the apramycin resistance gene from total DNA isolated from *S. chromofuscus*-SET152, *S. chromofuscus*-ACC152, *S. chromofuscus*-ASA152, *S. chromofuscus*-PCC152 and *S. chromofuscus*-PSA152.

S. chromofuscus and all transformants were cultured in ISP2 liquid media for 48 h as seed culture. 200 μl of each of the seed cultures was innoculted into 50 ml of R2YE liquid and agar plates, herboxidiene production liquid media, ISP2 liquid and agar plates to observe

the morphology of *S. chromofuscus* and all transformants in different media. For isolation of compounds, 200 µl of each strain from seed culture was grown in ISP2 agar plates separately and incubated at 28ºC for 7 days. For extraction of blue pigment compound, sterilized double distilled water was used whereas for extraction of red pigment compound, ethyl acetate was used as extraction solvent from *S. chromofuscus* and all transformants. The pH of blue colored water extract was changed to pH 2–3 by which the blue colored compound was changed into pink precipitate. After centrifugation at 6,000 x g for 15 min, the precipitate was resuspended in methanol-chloroform (1:1) and recentrifuged. An equal volume of water was added to the supernatant causing phase separation, and the chloroform phase was collected and dried in vacuo. The precipitate was dissolved in methanol-chloroform (1:1), cleared by centrifugation, and applied to a Sephadex LH-20 column (100 ml) previously conditioned with methanol-water (1:1) containing 0.5% acetic acid. After elution of the column with the same solvent (150 ml), 150 ml of methanol (90% solution; containing 0.5% acetic acid) and 150 ml of methanol-chloroform (2:3) containing 0.5% acetic acid were subsequently applied as eluents. The methanol-chloroform fraction eluted major blue pigment fraction. After addition of water to this fraction, the chloroform phase was obtained which was again washed with water and then finally 3 volumes of hexane was added to precipitate γ-actinorhodin, which was collected by centrifugation and freeze-dried.

The ethylacetate extract of red pigment compounds, in each case, was evaporated and then concentrated with 1.0 ml of methanol. Red colored compounds were purified by silica gel column chromatography and then by preparative TLC. A 15 µl aliquot of the

purified compound was analyzed by TLC and HPLC analyses using a reverse-phase C-18 column with 100% acetonitrile (solvent B) and 0.05% TFA in distilled water, with a flow rate of 1 ml/min. Detection was carried out with a UV absorbance detector, monitoring at 236 nm. The blue pigmented compound was analyzed by measuring the optical density of the extracts at 633 nm using a UV spectrophotometer (Shimadzu, Japan) whereas the structure of one of the red compounds was determined by ^{1}H-NMR, ^{13}C-NMR, HMBC, HMQC, COSY, ROESY, NOESY and DOSY and the mass was determined by ESI/MS and MALDI-ToF-ToF-MS.

2.8.7 Isolation of nargenicin

The recombinant plasmids pNVmetK18, pNVACC18 and pNV18 were transformed into *Nocardia* sp. wild type by electroporation method to generate *Nocardia* sp. metK18, *Nocardia* sp. ACC18 and *Nocardia* sp. NV18 respectively. *Nocardia* sp. CS682 was incubated in 50 ml of brain heart broth for 4 days at 37°C as a seed culture. 100 µl of seed culture was transferred into 5ml of fresh brain heart broth and incubated at 37°C for 2 days. Cells were harvested, washed twice with 5 ml of ice-cold water, and then resuspended in 100 µl of ice-cold 10% glycerol. The suspension was transferred to a chilled electroporation cuvette (0.4 cm) and mixed with 10 µl of desired DNA. After pulsing at 12.25 kV/cm with an Electro Cell Porator (Bio Rad Micropulser), the suspension was added to 1ml of BHI broth and incubated for 2 h at 37°C. Cells were then plated onto brain heart agar plate containing neomycin (500 µg/ml) and incubated for 3–5 days at 37°C. Some colonies were

observed. These colonies were scratched on to another brain heart agar plates containing neomycin (500 µg/ml). When we checked the sensitivity of *Nocardia* sp. CS682, it showed growth up to 200 µg/ml neomycin. The strain could not grow beyond the concentration of 200 µg/ml neomycin when culture in brain heart. *Nocardia* sp. CS682 transformants showed well growth in brain heart media containing up to 500 µg/ml neomycin. Thus, the transformants were selected by antibiotic resistant selection method.

Nocardia sp. wild type and its transformants were seeded in 50 ml brain heart media for 5 days in shaking incubator at 37ºC. 500 µl of seed culture from each strain was inoculated into 80 ml of oat meal media and incubated at 37ºC for 9 days. The cell pellets were removed from each sample by centrifugation at 6000 x g and the supernatant was extracted with a double volume of ethylacetate. The ethylacetate extract was evaporated and then concentrated with 1.0 ml of methanol. A 15 µl aliquot of the extract was analyzed on HPLC analysis using a reverse-phase C-18 column (Mytstil RP-18) with Buffer A (0.05% TFA in water) and Buffer B (acetonitrile) at the flow rate of 1.0 ml/min. Detection was carried out with a UV absorbance detector monitoring at 236 nm. The major peak corresponding to nargenicin was confirmed by liquid chromatography/mass spectrometry (LC/MS) analysis. The amount of nargenicin produced by each strain was quantified (Cho *et al*., 2009) and compared. Nargenicin standard was used as a reference.

2.9 Comparative study of morphology of *Streptomyces* strains

To compare the morphology of *S. venezuelae* and its recombinant strains, all strains were grown in R2YE liquid media as a seed culture for 36 h. 100 µl of each strain from seed culture was grown in SPA and R2YE agar plates separately and incubated at 28ºC for 5 days. To compare the morphology of *S. peucetius* and its recombinant strains, all strains were grown in R2YE liquid media as a seed culture for 48 h. 100 µl of each strain from seed culture was grown in NDYE and R2YE agar plates separately and incubated at 28ºC for 5 days. To compare the morphology of *S. lividans* and its recombinant strains, all strains were grown in YEME liquid media as a seed culture for 48 h. 100 µl of each strain from seed culture was grown in YEME agar plates separately and incubated at 28ºC for 9 days. Similarly, to compare the morphology of *S. venezuelae* YJ028 and its recombinant strains, all strains were grown in R2YE liquid media as a seed culture for 48 h. 100 µl of each strain from seed culture was grown in SPA and R2YE agar plates separately and incubated at 28ºC for 5 days. Similarly, morphology of *S. chromofuscus* and all recombinant strains were compared in herboxidiene production liquid media, R2YE liquid media, ISP2 and R2YE agar plates. *S. chromofuscus* and all recombinant strains were cultured in ISP2 liquid media for 48 h as seed culture. 200 µl of each strain from seed culture was grown in herboxidiene production liquid media, R2YE liquid media, ISP2 and R2YE agar plates separately and incubated at 28ºC for 5−7 days.

2.10 Study of growth rate of *Streptomyces* strains

To study the growth rate, wild type and transformant strains of *S. venezuelae* were grown in SCM media at 28ºC after 36 h of incubation in R2YE seed media. The cell pellets were collected at intervals of 12 h by centrifuging 50 ml of culture broth of each strain at 6,000 × g. Cell pellets were washed with distilled water and dried at 72ºC in a vacuum oven to constant weight. Dried cell pellets were obtained for growth rate analysis.

2.11 Antibacterial assay

The antibacterial activity of compounds isolated from different strains was assayed by paper disc method against *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus* (Lee *et al*., 2006).

2.12 TLC assay

For routine TLC assays, silica-coated glass plates (Kieselgel 60) containing a UV-fluorescent indicator (Merck, Darmstadt, Germany) was used. For TLC assay of herboxidiene, $CHCl_3$:CH_3OH (9:1) solvent system was used. For blue pigmented compounds, benzene: acetic acid (9:1) and for red pigmented compounds CH_3OH:$CHCl_3$ (9:1) mobile systems were used separately. For TLC of nargenicin, hexane: ethylacetate (1:1) was used as mobile phase.

2.13 RNA sample preparation and RT-PCR analysis

For total RNA isolation, *S. venezuelae* and all recombinant strains were cultured in APM medium. 5 ml aliquots of each culture at about 48−72 h were suspended in RNA protect Bacteria Reagent (Qiagen) for 5 min. An RNeasy Mini kit (Qiagen) was used for RNA isolation according to the instructions supplied by the manufacturer. Contaminant DNA in the sample was eliminated by using RNase-free DNase (Qiagen) and verified by PCR analysis with the RNA as the template. The total RNA concentration and purity were determined by measuring the optical density at 260 nm and 280 nm in a spectrophotometer. RT-PCR was performed with a QuantiTech SYBER Green RT-PCR Kit (Qiagen). Primers used for RT-PCR analysis are listed in **Table 2-3**. An equal amount of RNA (1.5 μg) was used for RT-PCR in every case. The reaction conditions were as follows: first-strand cDNA synthesis at 50ºC for 30 min; initial denaturation at 95ºC for 15 min; and 45 cycles of 1 min at 94ºC, 1 min at 63ºC, and elongation at 72ºC for 2 min. RT-PCR products were electrophoresed on 1% agarose gel and visualized using ethidium bromide staining. Negative controls were carried out with Taq DNA polymerase without reverse transcripts in order to confirm that the amplified products were not derived from chromosomal DNA that could have contaminated the RNA preparations. The 16S rRNA gene from *S. venezuelae* was used as a positive internal control. For further confirmation, the results obtained were cross-checked by running RT-PCR with decreased PCR cycles (i.e., 30 cycles).

2.14 Extraction and analysis of acyl-CoA

Intracellular acetyl-CoA and malonyl-CoA were extracted and quantified by following method described elsewhere (Park *et al*., 2007). 800 µl of a silicone oil mixture (AR200:DC200, 2:1, δ = 1.01) was added to a 2 ml microcentrifuge tube kept on ice, containing 500 µl 15% (w/v) TCA and then 800 µl cell suspension (rapidly drawn from the evenly mixed flask after 2 and 4 days) was added carefully to the silicone oil layer without perturbing it. The tube was centrifuged at 20,000 × g for 5 min at 4ºC, and 300 µl of the TCA extract (60% of the original cell extract) was immediately drawn using a Pasteur pipette. Each extract was eluted through an OASIS HLB SPE cartridge under vacuum. Before elution, the cartridge was conditioned with 3 ml methanol followed by 3 ml 0.15% TCA. The cell extract was then applied to the cartridge followed by 2 ml each 0.15% TCA and n-hexane. The CoA-esters were eluted two times with 0.5 ml of methanol-NH_4OH (99:1, v/v), evaporated to dryness at room temperature by vacuum centrifugation and kept in a freezer until analysis. The solution was reconstituted to 100 µl with water and 15 µl was subjected to HPLC analysis. A Hypersil BDS C-8 column was eluted isocratically with 100 mM potassium phosphate buffer (pH 7.4) and methanol (95:5 v/v) for 30 min at 1 ml/min. Peaks were detected at 254 nm. Authentic acetyl-CoA and malonyl-CoA were used as references. The amounts of acetyl-CoA and malonyl-CoA were averaged from three separate cultivations and extractions.

Chapter III

Heterologous expression of *metK1-sp* and *afsR-sp* in *Streptomyces venezuelae* for the production of pikromycin

3.1 Background

Pikromycin was the first isolated macrolide antibiotic and got its name from apparent bitter taste (Brockmann and Hekel, 1951). Pikromycin (**Fig. 3-1**), a 14-membered macrolide antibiotic, is structurally related to the semi-synthetic ketolide antibiotics, which are active against multidrug-resistant respiratory pathogens (Agouridas *et al*., 1998). Due to the wide range of biological activities, the biosynthesis of the pikromycin series of antibiotics has attracted significant interest. Pikromycin was initially isolated from *S. venezuelae* ATCC 15439. However, low levels of pikromycin production have led to the development of a strain genetically engineered to produce higher levels of pikromycin.

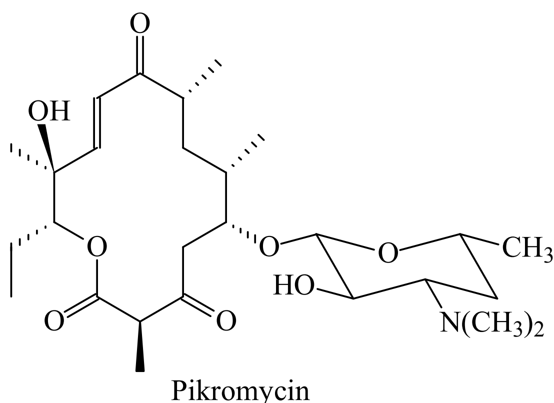


Figure 3-1. Structure of pikromycin.

Many of the previous studies on the enhancement of secondary metabolites were focused on either the homologous or heterologous expression of positive regulators. Among the positive regulators, S-adenosylmethionine synthetase (MetK) and a global regulatory

gene, *afsR*, were previously reported to be involved in the enhanced production of various secondary metabolites from different *Streptomyce* species (Lee *et al*., 2002; Kim *et al*., 2003; Okamoto *et al*., 2003; Wang *et al*., 2007). MetK plays an important role in the conversion of ATP and L-methionine to S-adenosyl-L-methionine (SAM), which acts as a major methyl group donor for numerous transmethylation reactions. Furthermore, SAM regulates antibiotic biosynthesis in a manner independent of its role as a methyl donor, in which it acts as a direct intracellular signaling molecule for *Streptomyces* (Zhao *et al*., 2006). It also activates the transcriptional activators responsible for the induction of antibiotic synthetic genes, thereby increasing the production of antibiotics (Kim *et al*., 2003). Recent reports have suggested that SAM induces several ABC transporters in order to modulate secondary metabolism and morphological development in *S. coelicolor* (Shin *et al*., 2007). On the other hand, *afsR* is a pleiotropic, global regulator that controls the production of secondary metabolites in *Streptomyces* species. AfsR is widely distributed as the AfsK-AfsR system in *Streptomyces* and influences the secondary metabolism and morphogenesis of the organism (Horinouchi, 2003). As a transcriptional activator, a*fsR* greatly enhances its DNA-binding activity toward the respective promoter region, thereby enhancing the transcriptional activation for the biosynthesis of secondary metabolites in *Streptomyces* (Tanaka *et al*., 2007).

On the basis of these findings and since *S. venezuelae* requires a short culture period (3–4 days) for metabolite production compared to other *Streptomyces* species, amenable to genetic manipulation and has high transformation efficiency, we heterologously expressed

these two genes, *metK1-sp* (GenBank accession no. CAJ43278) and *afsR-sp* (GenBank accession no. AJ786384) from *S. peucetius* ATCC 27952 (Parajuli *et al*., 2005) individually in *S. venezuelae* ATCC 15439 in order to analyze the production of pikromycin. RT-PCR was used to study the effects of the overexpression of these genes on the regulation of pikromycin biosynthetic gene clusters.

3.2 Results

3.2.1 Cloning and heterologous expression of *metK1-sp* and *afsR-sp*

With the aim to enhance the production of pikromycin, two positive regulator genes, *metK1-sp* and *afsR-sp*, which encode for the MetK protein and global regulatory protein, respectively were heterologously expressed in *S. venezuelae* wild strain. Both *metK1-sp* (1.2 kb) and *afsR-sp* (2.9 kb) were amplified from *S. peucetius* using the metK1-F/metK1-R and *afsR*-F/*afsR*-R primers respectively. The PCR products of *metK1-sp* and *afsR-sp* were separately cloned into pIBR25 to construct recombinant plasmids pSIBR and pGIBR respectively as described in the materials and methods section (**Fig. 3-2**). The recombinants pSIBR and pGIBR were then transformed into *S. venezuelae* by protoplast transformation method to generate *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR respectively.

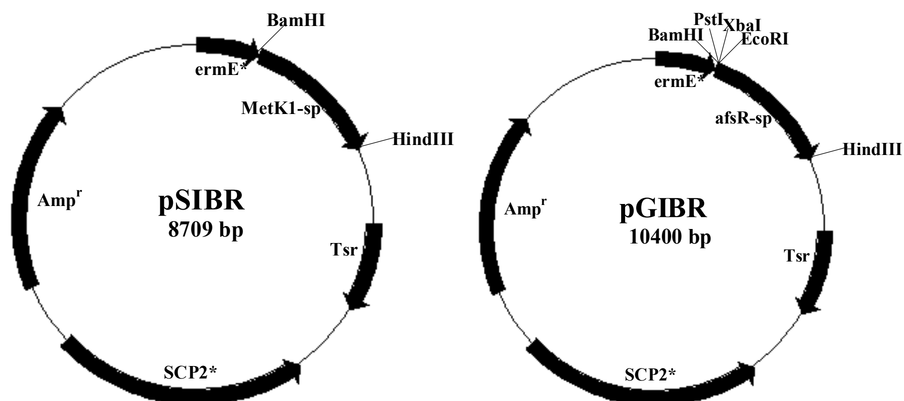


Figure 3-2. Recombinant Plasmids. (a) pSIBR, *metK1-sp* from *S. peucetius* cloned into the pIBR25. (b) pGIBR, *afsR-sp* from *S. peucetius* cloned into the pIBR25.

3.2.2 Effect of heterologous expression of *metK1-sp* and *afsR-sp* on pikromycin production

Whether the heterologous expression of two activator genes separately activated the production of pikromycin in *S. venezuelae* or not, the pikromycin was extracted from *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR under similar conditions and compared with that of *S. venezuelae*. Pikromycin produced by these strains were analyzed by HPLC (**Fig. 3-4**) and LC/MS analyses as described in materials and methods. It was found that production of pikromycin was enhanced by 1.6-fold in *S. venezuelae*/pSIBR and 2.6-fold in *S. venezuelae*/pGIBR in comparison with pikromycin production by *S. venezuelae* (**Fig. 3-5**). The biological activity of the pikromycin obtained from *S. venezuelae*, *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR were tested against *B. subtilis*, and the diameter of the inhibition zones in the two mutants showing the least growth were indicative of high levels of

pikromycin by transformants than that by *S. venezuelae* (**Fig. 3-3**). Furthermore, pikromycin produced by *S. venezuelae* wild type, *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR was isolated and analyzed at different time intervals and found that all these strains produced maximum pikromycin around 48−60 h (**Fig. 3-6**). These results revealed that *metK1-sp* and *afsR-sp* had a positive influence on the production of pikromycin.

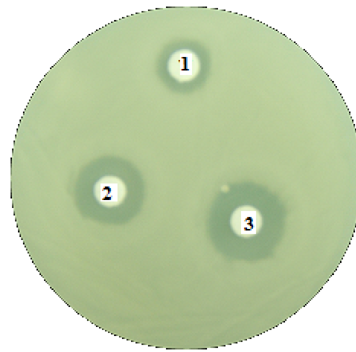


Figure 3-3. Antibacterial activity assay of the isolated pikromycin against *B. subtilis*. Isolates from *S. venezuelae* wild type (1), *S. venezuelae*/pSIBR (2) and *S. venezuelae*/pGIBR (3).

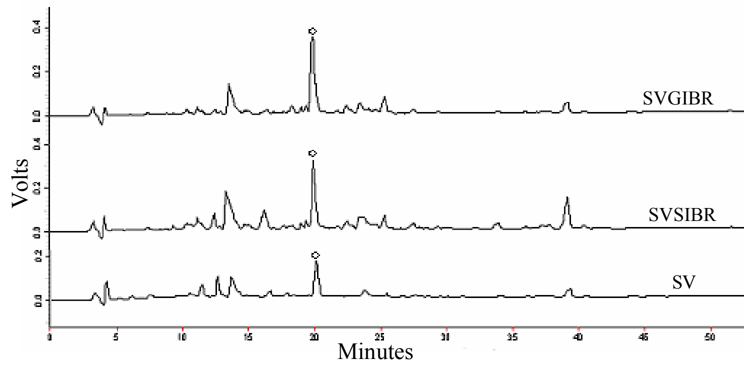


Figure 3-4. Typical HPLC traces for pikromycin. Peak corresponding to pikromycin is indicated by a circle (Retention time, 20 min), and the pikromycin peaks in every case were verified by LC/MS analysis. Note*:* SV; *S. venezuelae* wild type, SVSIBR; *S. venezuelae*/pSIBR and SVGIBR: *S. venezuelae*/pGIBR.

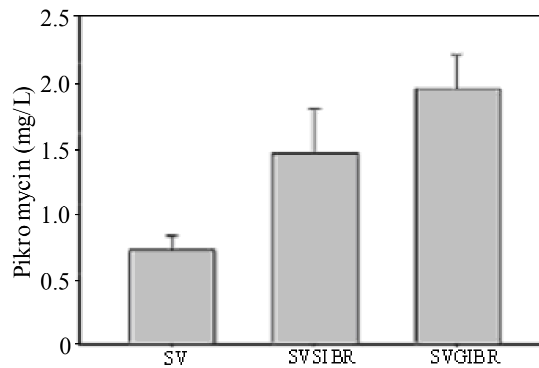


Figure 3-5. Comparison of pikromycin production from *S. venezuelae* wild type, *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR. Pikromycin was extracted from each strain, after being grown at 28ºC for 60 h in SCM medium, and analyzed by HPLC and LC/MS analysis. The experiment was averaged from 4 different extractions.

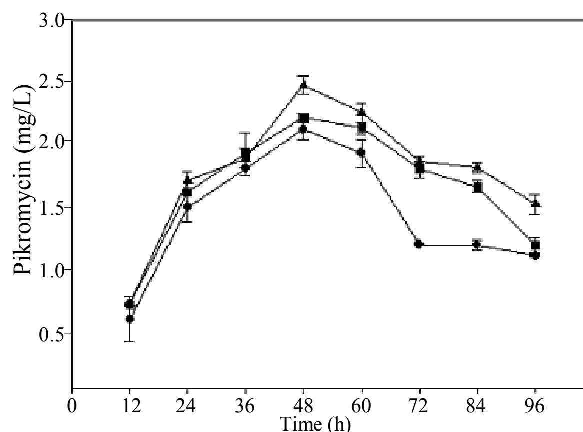


Figure 3-6. Monitoring production of pikromycin**.** Isolation of the compound was carried out at equal time intervals and quantification was performed via HPLC analysis. Closed circle, *S. venezuelae* wild type; closed square, *S. venezuelae*/pSIBR; and closed triangle, *S. venezuelae*/pGIBR.

3.2.3 Effect of heterologous expression of *metK1-sp* and *afsR-sp* on growth rate

To study whether enhanced production of pikromycin by two mutant strains were due to the increased growth rate, the growth rate of the *S. venezuelae*, *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR were analyzed at the time interval of 12 h as described in materials and methods. From the experiment, we found that *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR had higher growth yields than that of *S. venezuelae*. Maximum growth was observed at 48–60 h (**Fig. 3-4**). There were no morphological differences among *S. venezuelae*/pSIBR, *S. venezuelae*/pGIBR and the wild-type strain when grown in R2YE and SCM media (data not shown). Thus, the heterologous expression of these genes affected the growth of the strain as well. However, the influence of *afsR-sp* on the growth rate as well as the production of pikromycin was greater than that induced by *metK1-sp*.

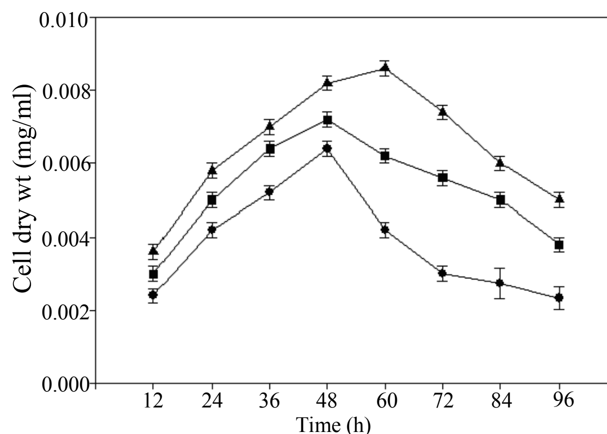


Figure 3-7. Comparison of the growth rate of *S. venezuelae* (closed circle), *S. venezuelae*/pSIBR (closed square) and *S. venezuelae*/pGIBR (closed triangle). Cell pellets were collected, washed and dried at 12 h intervals, starting from 12 h to 96 h.

3.2.4 RT-PCR analysis

Next to know whether the enhanced production of pikromycin by two mutant strains were only due to the increased growth rate or these activator genes activated the expression of pathway specific regulatory gene and/or biosynthetic genes, the influence of *metK1-sp* and *afsR-sp* on the expression levels of two genes, ketosynthase gene (*KS*) and activator gene (*pikD*), involved in the production of pikromycin were assayed by RT-PCR analysis as described in materials and methods. *KS* is a domain from PikAI that catalyses polyketide chain extension in modular multifunctional polyketide synthases (PKS) (Bisang *et al*., 1999), whereas PikD operates as a pathway-specific activator of the pikromycin biosynthetic gene cluster (Wilson *et al*., 2001). The RT-PCR results showed that the transcription levels of

both the *KS* and *pikD* genes were substantially increased in *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR when compared to that of *S. venezuelae* (**Fig. 3-5**).

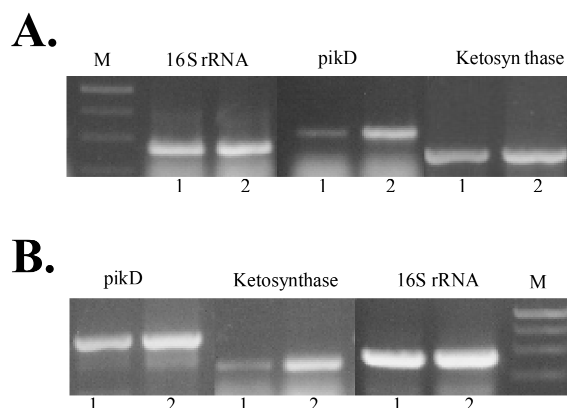


Figure 3-8. Quantitative RT-PCR analysis of *KS* and *pikD*, involved in pikromycin biosynthetic pathway using equal amount (5.54 ug) of total RNA isolated from (A) *S. venezuelae* and *S. venezuelae*/pSIBR and (B) *S. venezuelae* and *S. venezuelae*/pGIBR. 16S rRNA gene was used as internal control. M, DNA marker. In each case, Lane 1: total RNA from wild type and lane 2: total RNA from *S. venezuelae*/pSIBR or *S. venezuelae*/pGIBR.

3.3 Discussion

Pikromycin has attracted significant interest due to its activity against multidrug-resistant respiratory pathogens. *S. venezuelae* ATCC 15439 produces limited amount of pikromycin. The low levels of pikromycin production by wild type strain have led to the development of a genetically engineered strain to produce higher levels of pikromycin. Since, *S. venezuelae* is amenable to genetic manipulation as it possesses essential features such as short culture time, transformation efficiency etc, we chose the same strain to enhance the production of pikromycin.

To enhance the production of pikromycin from *S. venezuelae* wild strain, two positive regulators, *metK1-sp* and *afsR-sp* from *S. peucetius* were heterologously expressed in the producer strain. The results of the study revealed that the production of pikromycin was found to be enhanced by 1.6-fold in *S. venezuelae*/pSIBR and 2.6-fold in *S. venezuelae*/pGIBR in comparison to that produced by *S. venezuelae*. We presumed that the enhanced production of pikromycin in mutant strain was either due to increased growth rate or increased expression of pikromycin biosynthetic genes and/or pathway specific regulatory gene. When growth rate of wild and mutant strains was analyzed, *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR showed higher growth rate than that of *S. venezuelae*. In previous studies, the overexpression of *metK* stimulated the expression of the pathway-specific regulatory gene *act*II-ORF4 for the production of actinorhodin in *S. coelicolor* A3 (2) (Okamoto *et al*., 2003) and similarly AfsR, which serves as a transcriptional activator of afsS, also activated the pathway-specific regulatory gene *act*II-ORF4, in the actinorhodin biosynthetic gene cluster (Lee *et al*., 2002). Thus, strains overexpressing *metK1-sp* and *afsR-sp* were most likely to induce the biosynthetic pathway of pikromycin for the enhanced production of pikromycin. The influence of *metK1-sp* and *afsR-sp* on the expression levels of two genes, *KS* and *pikD*, involved in the production of pikromycin were assayed by RT-PCR analysis. *KS* is a domain from PikAI that catalyses polyketide chain extension in modular multifunctional polyketide synthases (PKS) (Bisang *et al*., 1999), whereas PikD operates as a pathway-specific activator of the pikromycin biosynthetic gene cluster (Wilson *et al*., 2001). The RT-PCR results showed that the transcription levels of both the *KS* and *pikD*

genes were substantially increased in *S. venezuelae*/pSIBR and *S. venezuelae*/pGIBR when compared to that of *S. venezuelae* (**Fig. 3-5**). In *S. venezuelae*, *metK* is located downstream of *pikD*, which might help to provide the methyl group in desosamine synthesis (Xue *et al*., 1998). Therefore, the increased transcripts of *pikD* might correspond to the enhanced production of desosamine and pikromycin. Studies on the biosynthesis of desosamine have consistently established DesVI (*N,N*-dimethyltransferase) as a SAM-dependent methyltransferase in the pikromycin biosynthetic gene cluster (Chen *et al*., 2002). The increased level of SAM production would apparently aid in the overproduction of desosamine by *desVI* and thereby enhance the production of pikromycin. Previously the reports showed that the enhanced expression of *metK* increases the intracellular level of SAM, which resulted in the overproduction of actinorhodin from *S. lividans* (Kim *et al*., 2003). Although the mechanism underlying the increased production of antibiotics with the increased intracellular level of SAM remains unclear, it has been proposed that SAM activates various transcriptional activators, which are responsible for the induction of antibiotic synthetic genes or serves as a methyl donor directly to the antibiotics. Similarly, *afsR* is a global regulator gene that seems to control the secondary metabolism and morphological and physiological differentiation in *Streptomyces* (Umeyama *et al*., 2002). Moreover, *afsR-sp* functioned as a transcriptional activator in order to regulate the production of secondary metabolites in *Streptomyces* species (Parajuli *et al*., 2005). Heterologous expression of *afsR-sp* in *S. venezuelae*/pGIBR enhanced the transcript levels of the *KS* and *pikD* pikromycin biosynthetic genes, resulting in the overproduction of

pikromycin. Although the exact mechanism of the increased production of antibiotics should be further elucidated, we found that the *metK1-sp* and *afsR-sp* genes influenced the stimulation of the biosynthetic genes to overproduce pikromycin through transcriptional activation in *S. venezuelae*.

In conclusion, *metK1-sp* and *afsR-sp* act as the positive regulators in the biosynthesis of pikromycin and it was found that the influence of *afsR-sp* on the growth rate as well as the production of pikromycin was greater than that induced by *metK1-sp*.

Chapter IV

Identification and functional characterization of an *afsR* homolog regulatory gene from *Streptomyces venezuelae* ATCC 15439

4.1 Background

S. venezuelae ATCC 15439 is a producer of several macrolide antibiotics that include the 12-membered polyketides methymycin, neomethymycin, and novamethymycin, and the 14-membered polyketides narbomycin, pikromycin, neopikromycin, and novapikromycin (Lee *et al*., 2006). Usually, genes for the production of individual secondary metabolites are arranged in clusters, and most but not all of these clusters contain pathway-specific regulatory genes whose expression frequently depends on genes that are required for the production of several secondary metabolites. The production is activated when the transcription of the pathway-specific regulators reach a threshold in the cells in response to specific environmental and physiological signals (Umeyama *et al*., 2002)

Interestingly, a number of regulators exert pleiotropic effects in *Streptomyces.* AfsR is one of these regulators that acts as a transcriptional factor in both the regulation of secondary metabolism in *S. coelicolor* A3 (2) and morphological differentiation in *S. griseus*. Moreover, AfsR is a protein belonging to the *Streptomyces* antibiotic regulatory protein (SARP) family, which consists of three major functional domains: an N-terminal SARP domain, a central ATPase domain, and a C-terminal tetratricopeptide repeat (TPR) domain. An example of SARPs includes ActII-ORF4, which functions in actinorhodin production in *S. coelicolor* A3(2) (Arias *et al*., 1999). Transcriptional activation by SARPs is expected to occur via an interesting and novel mechanism. As a transcriptional activator, AfsR is phosphorylated on its threonine residues by a protein serine/threonine kinase, AfsK, which enhances its DNA-binding activity and causes it to bind the promoter elements, including -

35 of *afsS*, thus resulting in activation of *afsS* transcription. AfsS then activates transcription of ActII-ORF4, a pathway specific transcriptional activator in the actinorhodin biosynthetic gene cluster, in an unknown way. Subsequent studies have shown that the AfsK/AfsR system is widely distributed in *Streptomyces* and influences secondary metabolism and morphogenesis (Horinouchi, 2003; Rajkarnikar *et al*., 2007). It has been reported that *afsR* overexpression led to the overproduction of the pigmented antibiotics actinorhodin and undecylprodigiosin and A-factor in *S. lividans*. Moreover, the *afsR* deletion mutant could not produce a detectable amount of actinorhodin (Kim *et al*., 2007).

In this study, we reported the identification and functional characterization of an *afsR* homolog global regulatory gene, designated as *afsR-sv*, from *S. venezuelae* ATCC 15439 and presented evidences that the *afsR-sv* is involved in the regulation of pikromycin biosynthesis. Furthermore, we also observed that *afsR-sv* influences the production of doxorubicin in *S. peucetius* and actinorhodin in *S. lividans* TK24.

4.2 Results

4.2.1 Identification of *afsR* homolog gene in *S. venezuelae*

A cosmid library of *S. venezuelae* was constructed using Super-Cos I, and screening was carried out by Colony hybridization, Restriction mapping and Southern hybridization using 600 bp PCR fragments as probe as described in the materials and methods. Sequencing analysis of the 5 kb DNA fragment of the cosmid using the available online softwares revealed an open reading frame (ORF) that constitutes 3,168 nucleotides, where ATG and

TGA were assigned as putative start and stop codons respectively. The start codon was preceded by the putative ribosome binding site (GGGGGAG). Homology searches for the ORF showed a high degree of homology to afsR encoding global regulatory genes from *S. coelicolor* and other *Streptomyces*. This *afsR* homolog gene of *S. venezuelae* was designated as *afsR-sv*. In fact, AfsR-sv encodes 1,056 amino acids with ATP binding sites (A-type: 407GIGGVGKT414; and B-type: 485LVLLD489) and a catalytic domain (440AEPET465). AfsR-sv exhibited high sequence homology with AfsR from other *Streptomyces* species (**Table 4-1**). Conserved domain analysis of AfsR-sv from NCBI and Pfam 22.0 revealed the presence of major functional domains of the *Streptomyces* antibiotic regulatory protein (SARP) family of transcriptional activators, including an N-terminal SARP domain, a bacterial transcriptional activation domain (BTAD), an NB-ARC domain and a C-terminal tetratricopeptide repeat (TPR) domain (**Fig. 4-1**). In addition to these major functional domains, AfsR-sv possesses two additional domains: DnrI, a DNA binding transcriptional activator of the SARP family (Sheldon *et al*., 2002), and MalT, an ATP-dependent transcriptional activator of the LuxR family (Richet *et al*., 1989; Schreiber *et al*., 1999). Owing to the presence of major functional domains that are characteristics of the SARP family, *afsR-sv* could be classified as a member of the SARP family of transcriptional activators and considered to function as a global regulator of secondary metabolites in *S. venezuelae*.

Table 4-1. Sequence comparison of AfsR-sv with other AfsRs

Gene	Organism	Genebank accession number	Number of amino acids	Identity to AfsR-sv	Similarity to AfsR-sv
afsR-sv	*S. venezuelae*	ABR08660	1056	100	100
afsR-sp	*S. peucetius*	CAH10136	982	75	82
afsR	*S. lividans*	ACJ04045	993	72	79
afsR	*S. coelicolor*	BAA14186	993	73	79
afsR-g	*S. griseus*	BAA83790	974	72	81
afsR-av	*S. avermitilis*	AAC68681	337	63	75

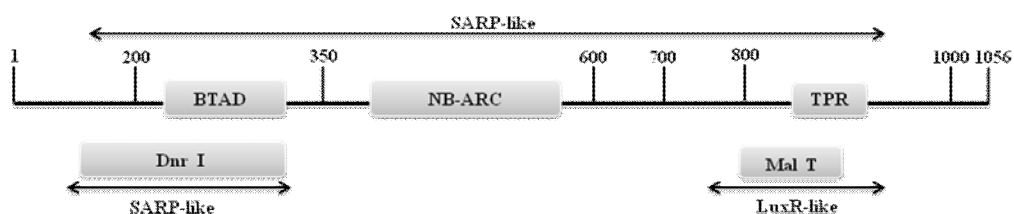


Figure 4-1. Domain analysis of AfsR-sv. AfsR-sv possesses the major functional domains of the *S.* antibiotic regulatory protein (SARP) family. N-terminal: SARP domain, a bacterial transcriptional activation domain (BTAD), an NB-ARC domain, and a DNA binding transcriptional activator of the SARP family (DnrI). C-terminal: tetratricopeptide repeat (TPR) domain and ATP dependent transcriptional activator of the LuxR family (MalT).

4.2.2 *afsR-sv* overexpression enhances the growth as well as the production of pikromycin

To study whether or not *afsR-sv* influences the production of pikromycin in *S. venezuelae*, *afsR-sv* was overexpressed in the producer strain. For this, *afsR-sv* was PCR amplified from genomic DNA of *S. venezuelae* and cloned into integration vector pSET152 to construct pASV152. The recombinant pASV152 was introduced into *S. venezuelae* wild type generating *S. venezuelae*/pASV152 as described in the materials and methods. Similarly, pSET152 was also introduced to generate *S. venezuelae*/pSET152. The production of pikromycin from *S. venezuelae* and transformants was compared at identical conditions. *S. venezuelae*/pASV152 showed a higher growth rate as compared to that of wild-type strain (**Fig. 4-2**). HPLC and LC/MS analyses of the extracts isolated from 50 ml culture broth of *S. venezuelae*, *S. venezuelae*/pSET152, and *S. venezuelae*/pASV152 after 60 h incubation at 28ºC revealed that *S. venezuelae*/pASV152 produced more pikromycin in SCM liquid medium as compared to that of *S. venezuelae* and *S. venezuelae*/pSET152. *S. venezuelae*/pASV152 exhibited an approximately 4.85-fold increase in pikromycin production when compared to wild-type (**Fig. 4-3**). Similarly, analysis of the production of pikromycin after various culture periods (12 to 96 h) revealed that the rate of pikromycin production by *S. venezuelae*/pASV152 at 60 to 96 h was higher than that of the wild-type strain (**Fig. 4-4**).

When the growth rate of the *S. venezuelae*, *S. venezuelae*/pSET152 and *S. venezuelae*/pASV152 were analyzed at the time interval of 12 h as described in materials

and methods it was found that *S. venezuelae*/pASV152 exhibited higher growth rate than that of *S. venezuelae* and *S. venezuelae*/pSET152. However, there were no morphological differences among *S. venezuelae*, *S. venezuelae*/pSET152 and *S. venezuelae*/pASV152 when grown in R2YE and SCM media (data not shown).

These results clearly demonstrated that overexpression of *afsR-sv* increased the production of pikromycin in *S. venezuelae* during the stationary phase.

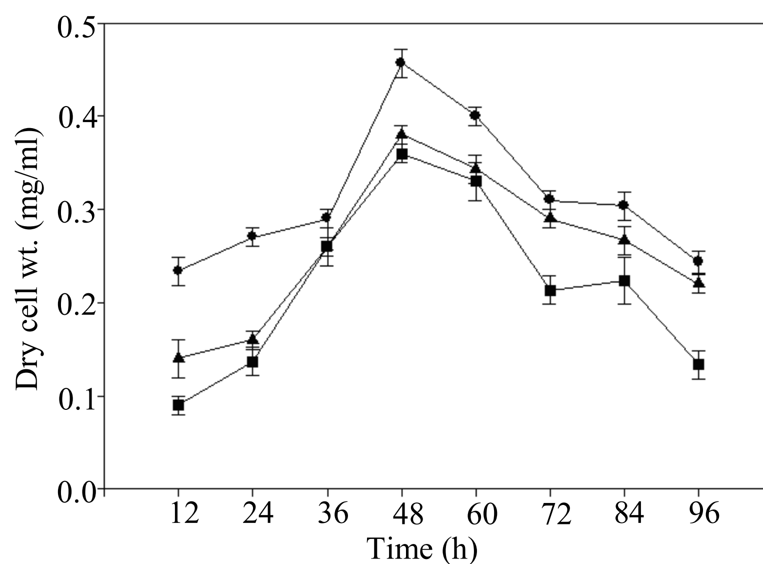


Figure 4-2. Comparison of the growth rate of *S. venezuelae* (closed square), *S. venezuelae*/pSET152 (closed triangle) and *S. venezuelae*/pASV152 (closed circle). Bars above the columns indicate standard errors.

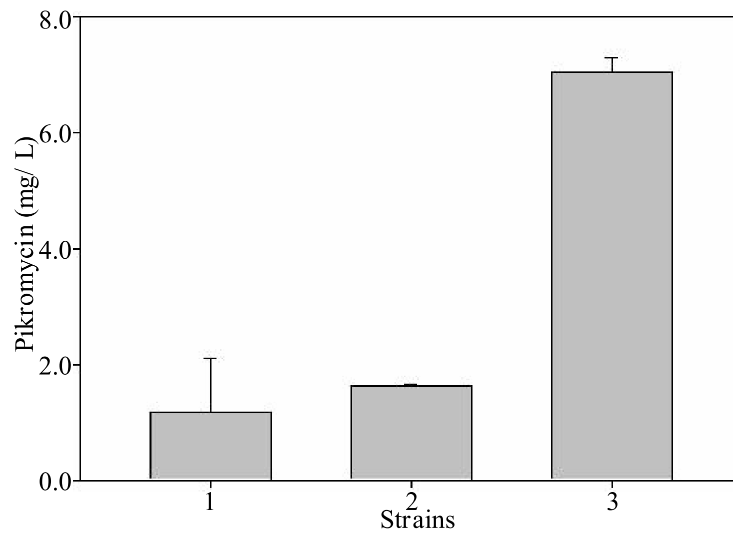


Figure 4-3. Effect of *afsR-sv* on the production of pikromycin. A. Pikromycin produced by *S. venezuelae* ATCC 15439 (1) and its transformants, *S. venezuelae*/pSET152 (2) and *S. venezuelae*/pASV152 (3) were analyzed. The amount of pikromycin produced by *S. venezuelae*/pASV152 was approximately 4.85 times greater than that produced by the wild-type strain. Bars above the columns indicate standard errors.

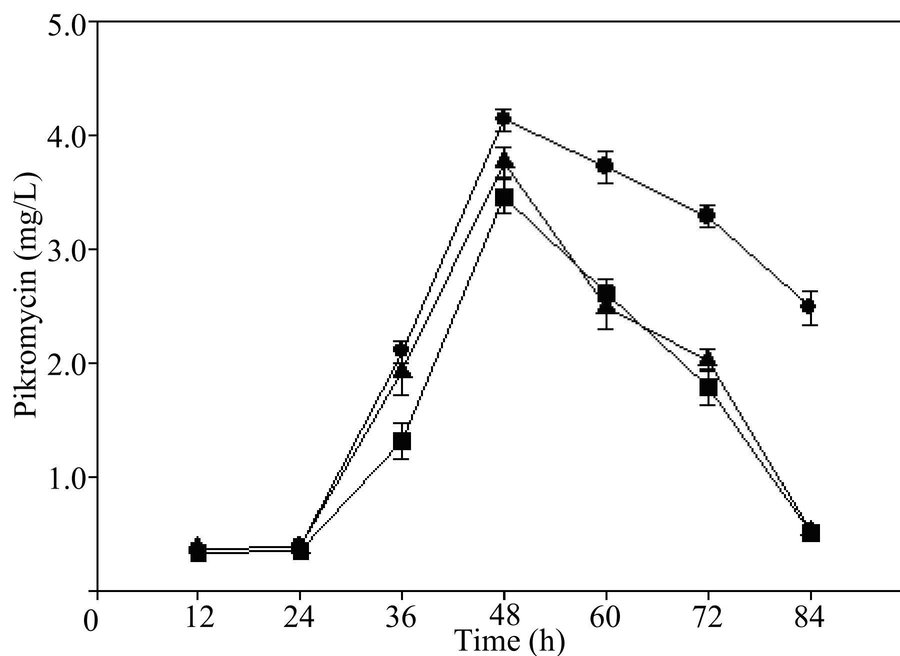


Figure 4-4. Comparison of influence of *afsR-sv* on the production of pikromycin. Pikromycin production by *S. venezuelae* ATCC 15439 (closed square), *S. venezuelae*/pSET152 (closed triangle), and *S. venezuelae*/pASV152 (closed circle) at the indicated time intervals. The amount of pikromycin produced by *S. venezuelae*/ pASV152 was approximately 4.85 times greater than that produced by the wild type strain when pikromycin was extracted after 60 h of incubation from the respective strain. Bars above the columns indicate standard errors.

4.2.3 Effect of *afsR-sv* on antibiotic production in other *Streptomyces* species

To study the influence of *afsR-sv* on the production of antibiotics other than pikromycin, the *afsR-sv* under the control of the *ermE** promoter was heterologously

expressed in *S. peucetius* (doxorubicin producer) and *S. lividans* TK24 (actinorhodin producer). *S. peucetius*, *S. peucetius*/pIBR25, and *S. peucetius*/pASV25 were cultured in NDYE medium and doxorubicin was isolated and analyzed as described in the materials and methods section. The amount of doxorubicin produced by *S. peucetius*/pASV25 was approximately 8 times greater than that produced by the wild-type strain (**Fig. 4-5**). The production of doxorubicin from *S. peucetius* and transformants was compared at identical conditions.

S. lividans TK24, *S. lividans* TK24/pIBR25, and *S. lividans* TK24/pASV25 were cultured in YEME medium, and actinorhodin was extracted as described in materials and methods. The amount of actinorhodin produced by *S. lividans* TK24/pASV25 was approximately 1.5 times greater than that produced by the wild-type strain (**Fig. 4-7**). The production of actinorhodin from *S. lividans* TK24 and transformants was compared at identical conditions.

Similarly, analysis of doxorubicin and actinorhodin productions after various culture periods revealed that *S. peucetius*/pASV25 produced higher levels of doxorubicin around 72 h (**Fig. 4-6**) and *S. lividans* TK24/pASV25 produced higher levels of actinorhodin around 8 days when compared with respective wild-type strains (**Fig. 4-8**). These results clearly demonstrated that although the regulatory networking of *afsR-sv* in *Streptomyces* is not uniform, AfsR-sv acts as a positive regulator and increases the antibiotics production when heterologously expressed in different *Streptomyces.* Consistent with other AfsR, *afsR-sv* also exerts pleiotropic effects on the production of multiple secondary metabolites.

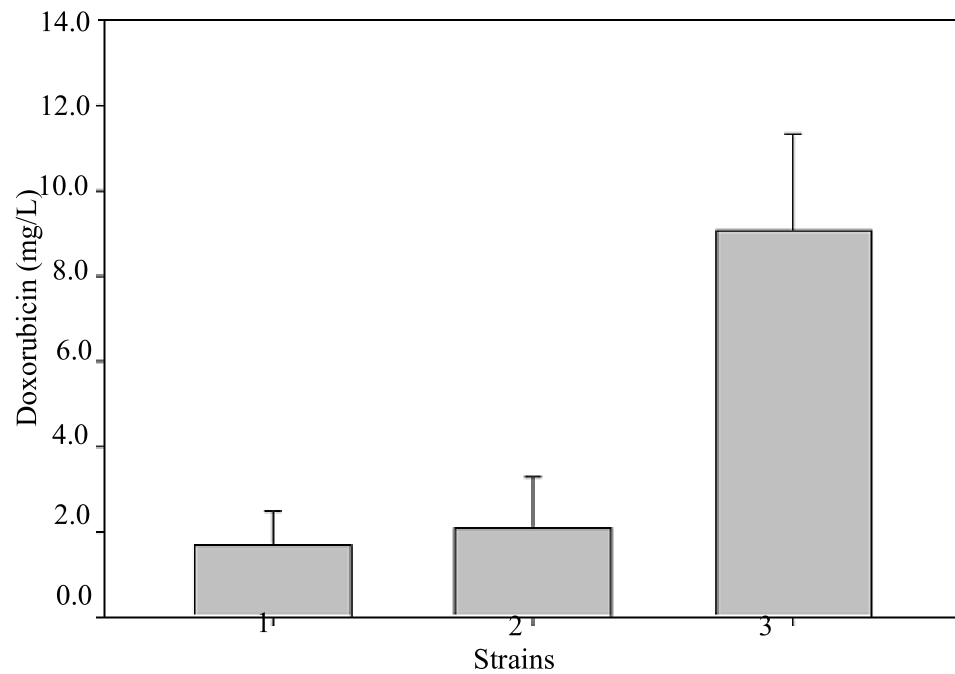


Figure 4-5. Effect of *afsR-sv* on the production of doxorubicin. Production of doxorubicin from *S. peucetius* ATCC 27952 (1), *S. peucetius*/pIBR25 (2) and *S. peucetius*/pASV25 (3). The amount of doxorubicin produced by *S. peucetius*/pASV25 was increased by approximately 8-fold, when doxorubicin was extracted after 72 h of incubation from the respective strain. Bars above the columns indicate standard errors.

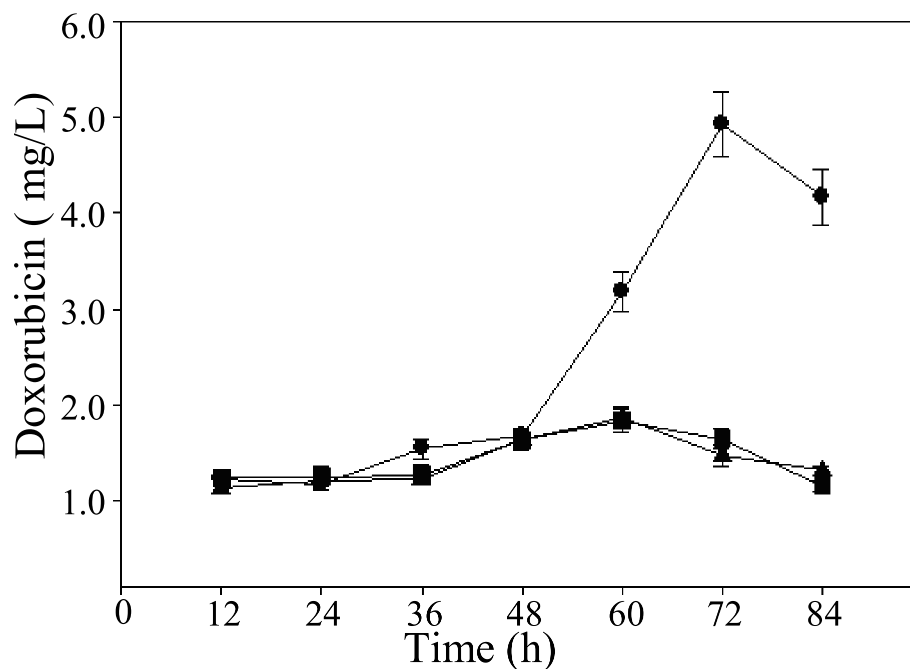


Figure 4-6. Comparison of production of doxorubicin from *S. peucetius* and transformants. Doxorubicin production by *S. peucetius* ATCC 27952 (closed square), *S. peucetius*/pIBR25 (closed triangle) and *S. peucetius*/pASV25 (closed circle) at the indicated time intervals. The amount of doxorubicin produced by *S. peucetius*/ pASV25 was increased by approximately 8-fold, when doxorubicin was extracted after 72 h of incubation from the respective strain. Bars above the columns indicate standard errors.

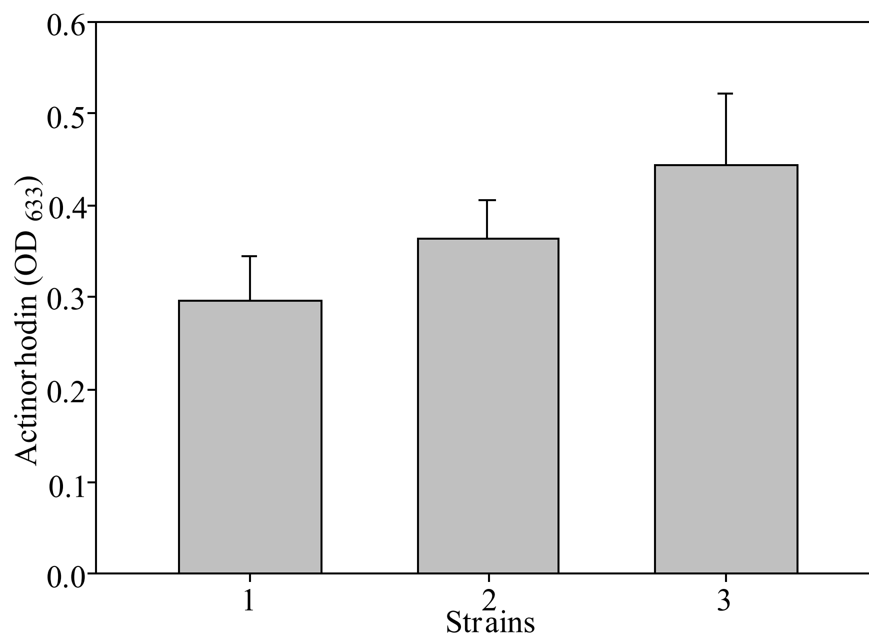


Figure 4-7. Effect of *afsR-sv* on the production of actinorhodin. Actinorhodin production by *S. lividans* TK24 (1), *S. lividans* TK24/pIBR25 (2) and *S. lividans* TK24/pASV25 (3). Actinorhodin production by *S. lividans* TK24/pASV25 was increased by approximately 1.5-fold, when actinorhodin was extracted after 8 days of incubation from the respective strain. Bars above the columns indicate standard errors.

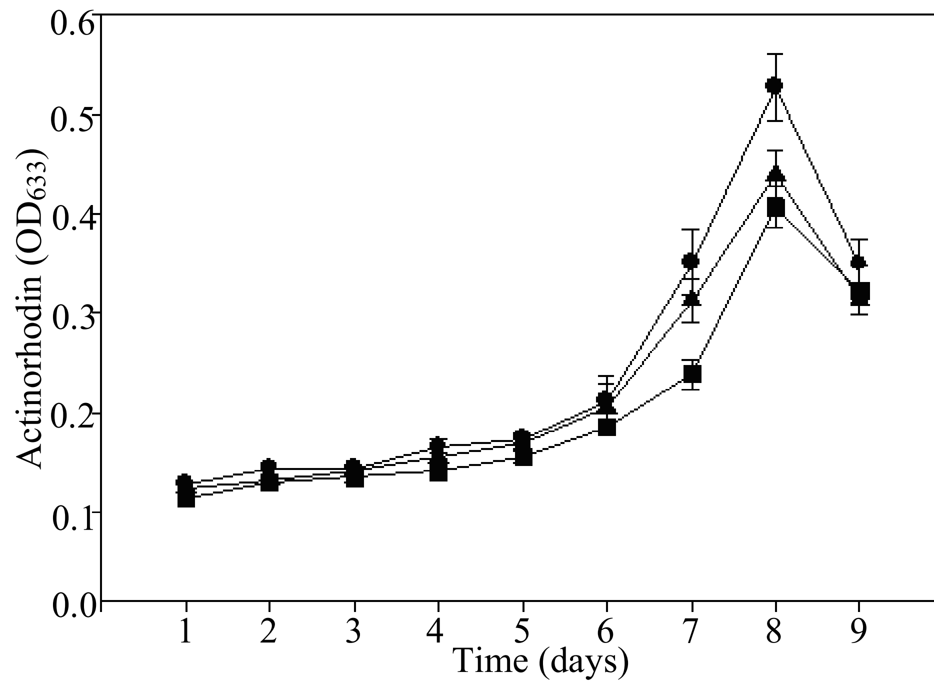


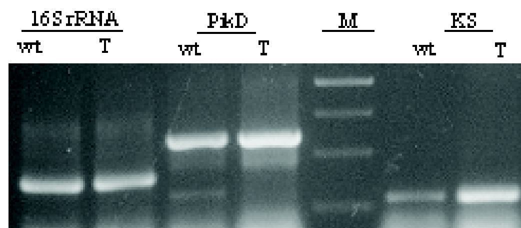
Figure 4-8. Comparison of production of actinorhodin from *S. lividans* TK24 and transformants. Actinorhodin production by *S. lividans* TK24 (closed square), *S. lividans* TK24/pIBR25 (closed triangle) and *S. lividans* TK24/pASV25 (closed circle) at the indicated time intervals. Bars above the columns indicate standard errors.

4.2.4 Transcriptional analysis

To study how *afsR-sv* affects the pikromycin biosynthesis, RT-PCR analysis was carried out to assess the expression levels of two representative genes: the ketosynthase gene (*KS*) and *pikD* (encoding the pathway specific activator of the pikromycin gene cluster) at 48 h and 72 h of growth. The results showed that the transcript levels of both *KS* and *pik*D were increased in the *afsR-sv* overexpressed strain (*S. venezuelae*/pASV152) when compared with *S. venezuelae* (**Fig. 4-9**) and this finding is consistent with the increased production of pikromycin. Moreover, to assess whether the regulation of *afsR-sv* in the *KS* and *pikD* biosynthetic genes is growth phase-dependent, RT-PCR was performed using multiple cultures according to the time course described for *S. venezuelae* and *S. venezuelae*/pASV152. The transcription of *KS* and *pikD* in *S. venezuelae*/pASV152 was observed to be higher from earlier growth phase to the later growth phase as compared to that of *S. venezuelae*. In both *S. venezuelae* and *S. venezuelae*/pASV152, the transcript levels of both genes was found to be maximum at around 48–72 h, after which the transcript level was found to be decreased, suggesting that the regulation of *afsR-sv* in the pikromycin biosynthetic gene cluster was growth-phase and time-dependent. In later growth phases, after around 84 h of growth, the transcription level of both *KS* and *pik*D was found to be decreased drastically in *S. venezuelae* whereas in case of *S. venezuelae*/pASV152, the transcription of these genes decreased slowly (**Fig. 4-10**). Thus, the influence of *afsR-sv* on

the transcription of *KS* and *pikD* was observed to be growth-phase-dependent. To confirm the enhanced expression of the *KS* and *pikD* by *afsR-sv,* the transcriptional analysis was further carried out by RT-PCR with decreased PCR cycles. Even when the RT-PCR was carried out with decreased PCR cycles, the transcript levels of both *KS* and *pikD* were found to be increased in the *afsR-sv* overexpressed strain than that in wild strain.

A



B

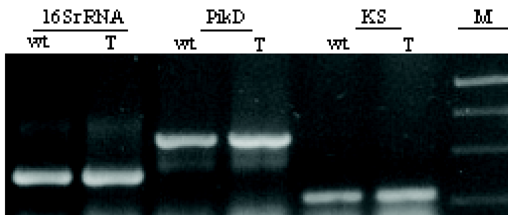


Figure 4-9. Transcriptional analysis of *pikD* and *KS* by RT-PCR in *S. venezuelae* and *S. venezuelae*/pASV152 strains at 48 h (A) and 72 h (B). RT-PCR was performed with equal amounts of RNA isolated from APM medium at the time indicated. RT-PCR was carried out without reverse transcriptase to confirm that the signals shown were derived from mRNA and not from contaminated genomic DNA. 16S rRNA, internal control; M, DNA marker; KS, ketosynthase; wt, *S. venezuelae*; T, *S. venezuelae*/pASV152.

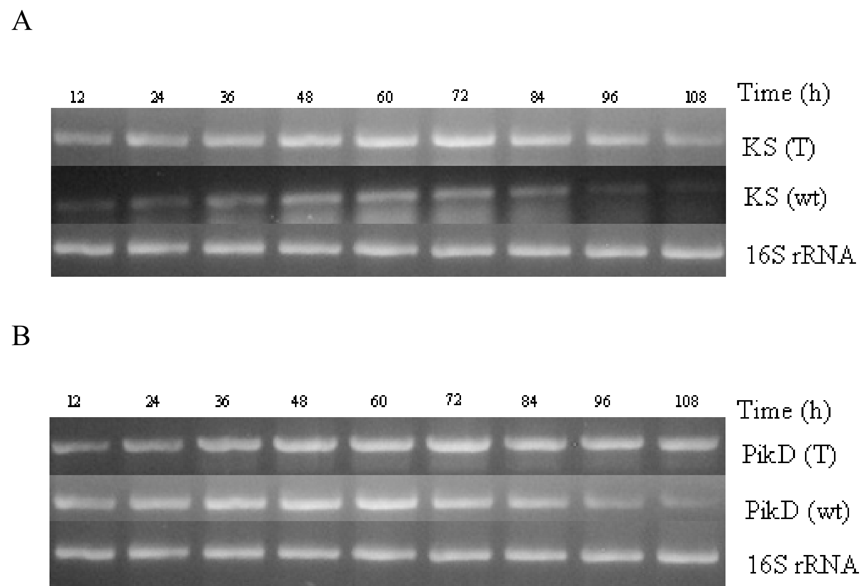


Figure 4-10. Time-dependent transcriptional analysis of *KS* (A) and *pikD* (B) by RT-PCR in *S. venezuelae* and *S. venezuelae*/ pASV152 strains. The transcription of both *KS* and *pikD* was found to be higher at around 48 to 72 h in *S. venezuelae*/ pASV152 as compared with that of *S. venezuelae.* The experiments were repeated three times. 16S rRNA, internal control; KS, ketosynthase; wt, *S. venezuelae*; T, *S. venezuelae*/pASV152.

4.3 Discussion

Many *Streptomyces* species harbor *afsR* or *afsR*-homolog global regulatory gene such as *S. coelicolor, S. peucetius, S. lividans, S. griseus* etc (Kim *et al*., 2006, Kim *et al*., 2001, Parajuli *et al*., 2005 and Umeyama *et al*., 1999). Sequencing analysis of 5 kb genome of *S. venezuelae* ATCC 15439 revealed the presence of one 3.1 kb open reading frame (ORF), designated as *afsR-sv*. The deduced product of *afsR-sv* (1,056 aa) was found to have high homology with the global regulatory protein, AfsR. Homology based analysis showed that *afsR-sv* represents a transcriptional activator belonging to the SARP family that includes an N-terminal SARP domain containing a bacterial transcriptional activation domain (BTAD), an NB-ARC domain, and a C-terminal tetratricopeptide repeat domain. The global regulatory genes, even though not closely linked to the biosynthetic genes, keep control over antibiotic biosynthetic pathways. These global regulatory genes have been found to play a significant role in secondary metabolism and morphological differentiation in *Streptomyces*. Among them, the *afsR-sp* gene from *S. peucetius* was found to regulate the production of doxorubicin (Parajuli *et al*., 2005) and *afsR-g* from *S. griseus* was found to be involved in morphological differentiation (Umeyama *et al*., 1999). In addition, the *afsR*2 from *S. lividans* was reported to highly stimulate two structurally unrelated antibiotics, actinorhodin and undecylprodigiosin, in both *S. lividans* and *S. coelicolor* (Kim *et al*., 2006, Kim *et al*., 2001). Consistent with these results, overexpression of *afsR-sv* in *S. venezuelae* increased pikromycin production by about 4.85-fold. Likewise, heterologous expression of *afsR-sv* resulted in increased production of actinorhodin by about 1.5-fold in *S. lividans* TK24 and

doxorubicin by about 8-fold in *S. peucetius*. In this context we observed a much higher level of doxorubicin production in *S. peucetius* when compared with the levels of pikromycin and actinorhodin production by *S. venezuelae* and *S. lividans* TK24 respectively. These findings indicate that several factors other than the *afsR-sv* are engaged to affect the production of secondary metabolites in *Streptomyces*.

As in the case of *S. coelicolor* A3(2), where AfsR shows pleiotropic effects on secondary metabolism, the effect of *afsR-sv* is also not uniform in regulatory networking in *Streptomyces*. RT-PCR assays demonstrated that *afsR-sv* functions as a positive regulator and is involved in the regulation of pikromycin biosynthesis in *S. venezuelae*. Overexpression of *afsR-sv* in *S. venezuelae* seemed to activate the transcription of ketosynthase and a pathway-specific regulator, both resulting in the overproduction of pikromycin. RT-PCR analysis also revealed that antibiotic production in *Streptomyces* species generally depends on the growth phase and involves the expression of physically clustered regulatory and biosynthetic genes. However, the mechanism of regulation by *afsR-sv* in *S. venezuelae* has yet to be elucidated. In conclusion, *afsR-sv* is a global regulatory gene in *S. venezuelae*, which belongs to the SARP family of transcriptional activators, regulates the pikromycin biosynthetic genes, and acts as a positive regulator of antibiotic production in *Streptomyces* strains.

Thus, in this study we identified and characterized an *afsR* homolog global regulatory gene, designated *afsR-sv*, from *S. venezuelae* and also studied the influence of *afsR-sv* in the production of different antibiotics from the respective strains.

Chapter V

Developing *Streptomyces venezuelae* as an efficient host to enhance heterologous production of polyketides

5.1 Background

Actinomycetes produce more than 10,000 drugs, many of which are polyketide derived compounds. However, wild type strains are not optimal hosts for high-level polyketide production as they tend to grow slowly and their cellular metabolic networks are not optimized for industrial production. An approach to polyketide overproduction is the metabolic engineering of a producer strain to develop a generic host into which polyketide synthase (PKS) genes and other essential genes could be readily transferred and functionally expressed (Olano *et al*., 2008). Alternatively, the polyketides can be produced in heterologous hosts which can facilitate analysis of the catalytic properties of polyketide-producing enzymes and also offer an excellent opportunity for precursor directed synthesis (Pfeifer and Khosla, 2001).

The usual heterologous host for polyketide production includes *E. coli*, *Bacillus subtilis*, *S. coelicolor, Saccharopolyspora erythraea*, *S. lividans* and *S. fradiae*. However, these heterologous hosts have slow growth rates and need relatively prolonged culture periods (6–9 days) to reach the maximum production level of metabolites. In contrast, *S. venezuelae* has recently been developed as a heterologous host (Yoon *et al*., 2002; Jung *et al*., 2003) that requires a short culture period (3–4 days) for metabolite production compared to other *Streptomyces* species. It is also amenable to genetic manipulation and has high transformation efficiency. These characteristics make *S. venezuelae* an attractive alternative system for rapid heterologous production of polyketides from bench-top genetic manipulation to product fermentation (Xue and Sherman, 2001).

Polyketides are assembled by a series of decarboxylative condensations of simple carboxylic acid precursors catalyzed by polyketide synthase complexes. The availability of biosynthetic precursors is a key factor determining the production of secondary metabolites. Thus, one of the metabolic engineering processes by which a particular metabolite can be overproduced is to alter the metabolic flux distribution of its different precursors. Actinorhodin production by *S. coelicolor* is an example of enhanced polyketide production by modification of its precursor supplies (Ryu *et al*., 2006) (**Fig. 5-1**). The overexpression of the genes *accA2*, *accB* and *accE*, coding for the different subunits of the enzyme ACC in *S. coelicolor* was sufficient to enhance carbon flux to malonyl-CoA, which is a precursor of actinorhodin together with acetyl-CoA, leading to a six-fold increase in actinorhodin production (Ryu *et al*., 2006).

Previous studies on the enhancement of secondary metabolites were focused on either homologous or heterologous expression of positive regulators. Among the positive regulators, S-adenosylmethionine synthetase and a global regulatory gene were previously reported to be involved in the enhanced production of various secondary metabolites from different *Streptomyces* species (Maharjan *et al*., 2008).

Thus, in this study, we developed four different *S. venezuelae* YJ028 mutant hosts. These newly developed *S. venezuelae* YJ028 mutant strains could be proved as an efficient heterologous host for the enhanced biosynthesis of different pharmaceutically important polyketide compounds. We engineered *S. venezuelae* YJ028 by expressing ACC, *metK1-sp* and *afsR-sp* so as to increase the malonyl-CoA pool to be directed towards various

polyketides that utilize aetyl CoA and malonyl CoA for the biosynthesis. ACC comprises *accA2* and *accBE* from *S. coelicolor*. Similarly, *S. venezuelae* YJ028 was also engineered by expressing propionyl CoA carboxylase (PCC), *metK1-sp* and *afsR-sp* so as to increase the methylmalonyl-CoA pool to be directed towards various polyketides that utilize propionyl CoA and methylmalonyl CoA for the biosynthesis. PCC comprises *accA1* and *pccBE* from *S. coelicolor* and *prpE* from *S. typhimurium.* ACC catalyses the biosynthesis of malonyl coenzyme A through ATP-dependent carboxylation of acetyl-CoA (Rodriguez *et al*., 2001; Tong, 2005) (**Scheme 5-1**) whereas PCC catalyses the biosynthesis of methylmalonyl coenzyme A by carboxylation of propionyl-CoA (Rodriguez *et al*., 1999) (**Scheme 5-2**).

To study the efficacy of the newly developed *S. venezuelae* host in heterologous production of various polyketides, we expressed type III polyketide synthase gene, *sp-rppA*, which encodes 1,3,6,8-tetrahydroxynaphthalene synthase (THNS) and analyzed the production of flaviolin.

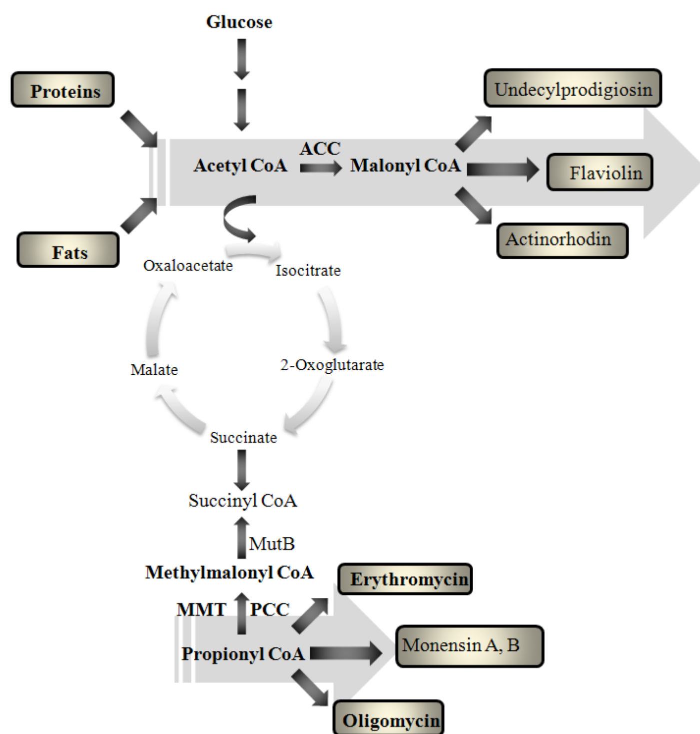
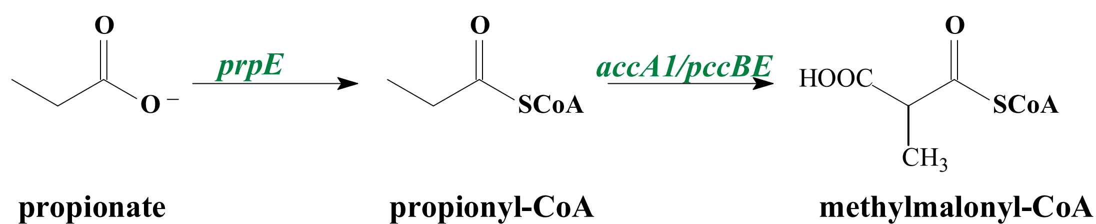


Figure 5-1. Representative diagram of fatty acid precursors and engineered steps involved in the biosynthesis of several secondary metabolites produced by actinomycetes. ACC, acetyl-CoA carboxylase; MMT, methylmalonyl-CoA transcarboxylase; PCC, propionyl-CoA carboxylase.

Scheme 5-1. Schematic representation of reaction catalyzed by ACC to convert acetyl-CoA into malonyl-CoA.



Scheme 5-2. Schematic representation of reaction catalyzed by PCC to convert propionate into methylmaonyl CoA.

5.2 Results

5.2.1 Construction of recombinant plasmids

The SAM synthetase gene (*metK1-sp*) was amplified from genomic DNA of *S. peucetius* ATCC 27952 using a pair of primers, metK1-spF/metK1-spF, and was cloned into the *Xba*I-*Eco*RI of pIBR25 to obtain pMetK25. Similarly, *afsR-sp* (2,949 bp) was also amplified from genomic DNA of *S. peucetius* ATCC 27952 using primer pairs, afsR-spF/afsR-spF, and cloned into the *Eco*RI-*Hin*dIII of recombinant pMetK25 to yield pMA25 as described in materials and methods. The *Xba*I-*Xba*I fragment, comprising *metK1-sp* and *afsR-sp*, was purified from pMA25 and cloned into pACC152 and pPCC152 to obtain pASA152 and pPSA152 respectively. The recombinant plasmids pACC152 and pPCC152 harboring ACC genes and PCC genes respectively were received from Prof. Yoon's lab. The recombinant pGG1 (Ghimire *et al*., 2008) was used to express the *sp-rppA* gene in *S. venezuelae* YJ028 (**Fig. 5-2**).

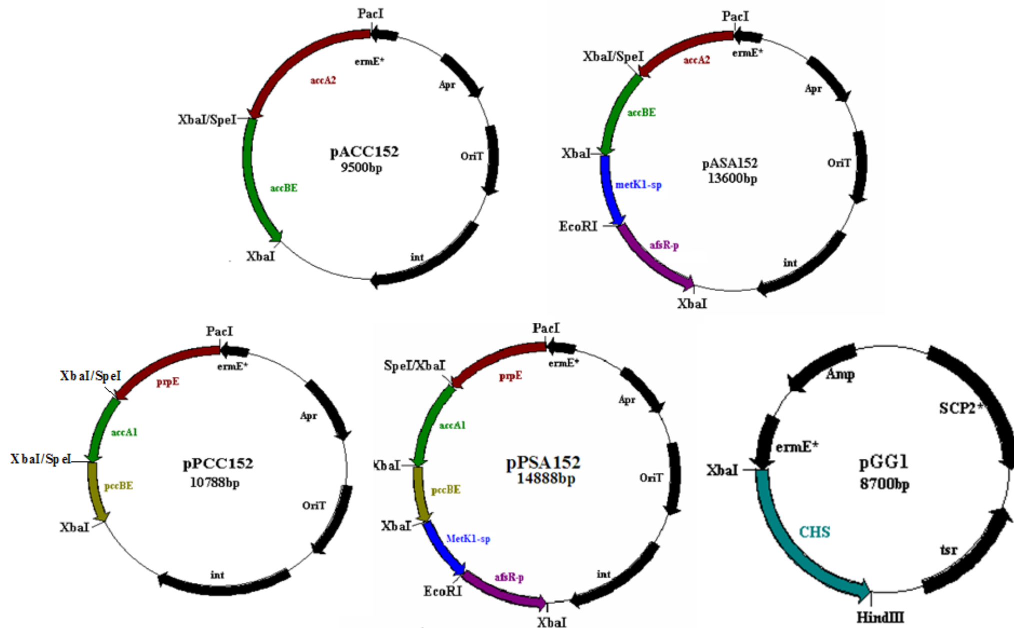


Figure 5-2. Recombinant plasmids used in this study.

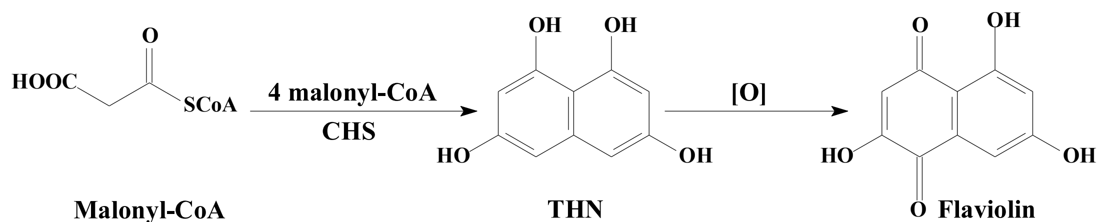
5.2.2 Expression of ACC and PCC in *S. venezuelae* YJ028

With the aim to increase the precursor pool to be directed towards various polyketides, we have chosen to increase the most common precursors, malonyl CoA and methylmalonyl CoA, which could be applied in the biosynthesis of many pharmaceutically important polyketide compounds. For this, ACC and PCC genes were separately expressed in *S. venezuelae* YJ028 by integrating pACC152, pASA152, pPCC152 and pPSA152 into the genomic DNA of *S. venezuelae* YJ028 generating *S. venezuelae* 28-ACC152, *S. venezuelae* 28-ASA152, *S. venezuelae* 28-PCC152 and *S.venezuelae* 28-PSA152 respectively following the standard protoplast transformation protocol (Kieser *et. al.,* 2000).

The integrative plasmid pSET152 was also integrated into genomic DNA of *S. venezuelae* YJ028 generating *S. venezuelae* 28-SET152 for control experiment. Transformants in each case were selected with apramycin (500 μg/ml). Integration of pSET152, pACC152 pASA152, pPCC152 and pPSA152 into chromosomal DNA of *S. venezuelae* YJ028 was confirmed by PCR of the apramycin resistance gene from total DNA isolated from *S. venezuelae* 28-SET152, *S. venezuelae* 28-ACC152 and *S. venezuelae* 28-ASA152, *S.venezuelae* 28-PCC152 and *S.venezuelae* 28-PSA152.

5.2.3 Expression of 1,3,6,8-tetrahydroxynaphthalene synthase (Sp-RppA)

To study the efficacy of newly developed *S. venezuelae* YJ028 hosts to produce polyketides heterologously, we expressed the type III polyketide synthase, Sp-RppA, from *S. peucetius* ATCC 27952 which encodes 1,3,6,8-tetrahydroxynaphthalene synthase (THNS). Sp-RppA catalyzes the condensation of five molecules of malonyl-CoA to form THN, which readily oxidizes to give 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin) (**Scheme 5-3).** We transformed pGG1 harboring *sp-rppA* (**Fig. 5-2**) into *S. venezuelae* 28-ACC152 and *S. venezuelae* 28- ASA152 generating *S. venezuelae* 28-ACC152/GG1 and *S. venezuelae* 28-ASA152/GG1 respectively. *S. venezuelae* YJ028 was also transformed with pGG1 to generate *S. venezuelae* 28-GG1 for comparative study. Transformants in each case were selected with apramycin (500 μg/ml) and thiostrepton (500 μg/ml). Transformation of pGG1 was confirmed by isolation and restriction enzyme digestion of plasmid from each strain.



Scheme 5-3. Schematic representation of reaction catalyzed by Sp-RppA (THNS) to convert five molecules of malonyl-CoA into flaviolin.

5.2.4 Analysis of flaviolin production

Flaviolin was isolated and analyzed from *S. venezuelae* YJ028, *S. venezuelae* 28-SET152, *S. venezuelae* 28-ACC152, *S. venezuelae* 28-pASA152, *S. venezuelae* 28-GG1, *S. venezuelae* 28-SET152-GG1, *S. venezuelae* 28-ACC152/GG1 and *S. venezuelae* 28-ASA152/GG1 and analyzed by HPLC (**Fig. 5-3**), LC/MS and GC/MS spectrophotometry as described in materials and methods. Our results showed that *S. venezuelae* 28-ACC152/ GG1 and *S. venezuelae* 28-ASA152/GG1 produced higher amounts of flaviolin than that of *S. venezuelae* 28-GG1. *S. venezuelae* 28-ACC152/GG1 and *S. venezuelae* 28-ASA152/ GG1 showed about 2-fold and 4-fold enhanced production of flaviolin as compared to *S. venezuelae* 28-GG1 (**Fig. 5-4**). Flaviolin was not detected from *S. venezuelae* YJ028, *S. venezuelae* 28-SET152, *S. venezuelae* 28-ACC152 and *S. venezuelae* 28-pASA152.

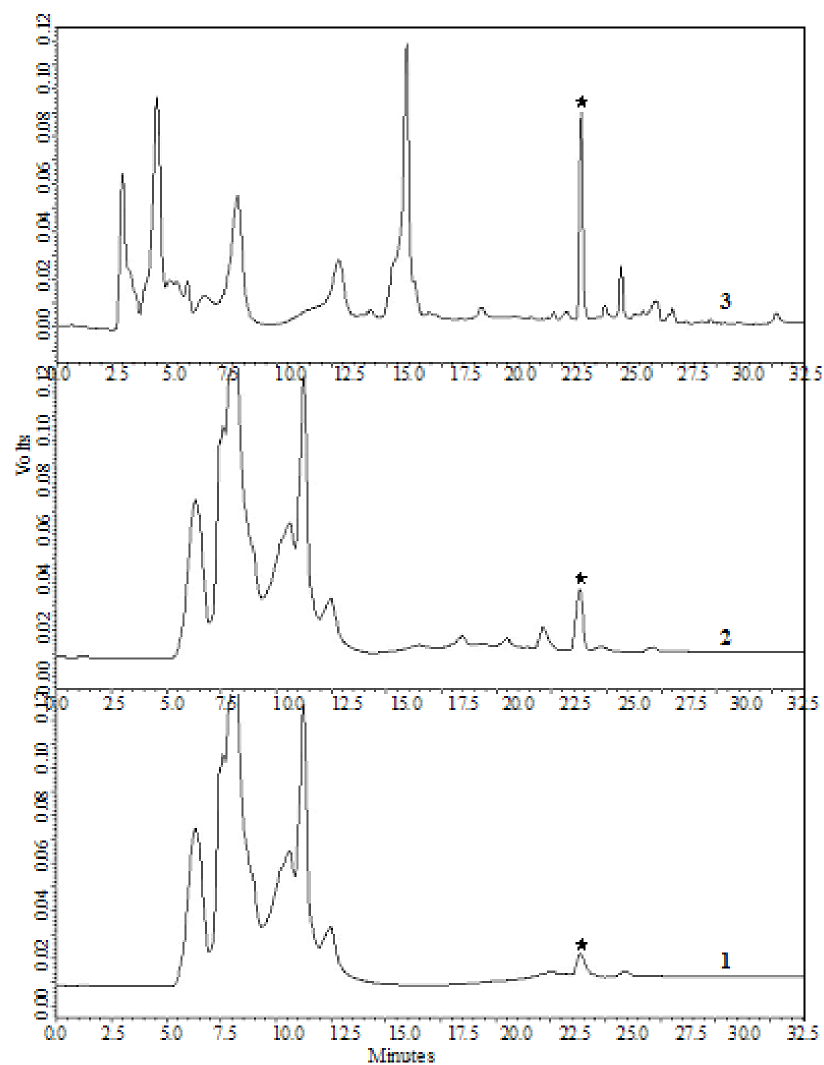


Figure 5-3. Typical HPLC chromatogram of flaviolin. (1) Compounds isolated from *S. venezuelae* 28-GG1; (2) Compounds isolated from *S. venezuelae* 28-ACC152/GG1; (3) Compounds isolated from *S. venezuelae* 28-ASA152/GG1. Peak corresponding to flaviolin is indicated by a star. The peak corresponding to flaviolin in every case was verified by LC/MS analysis.

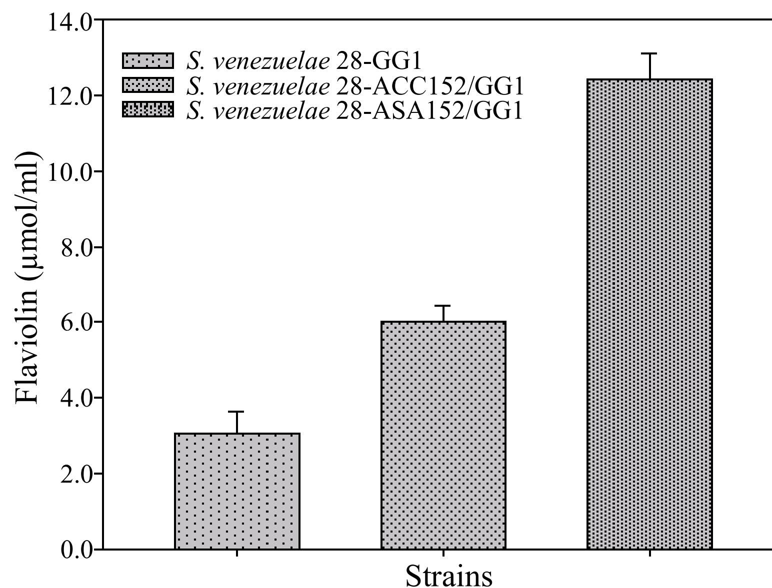


Figure 5-4. Comparison of flaviolin production by *S. venezuelae* 28-GG1, *S. venezuelae* 28-ACC152/GG1 and *S. venezuelae* 28-ASA152/GG1. Flaviolin was extracted from *S. venezuelae* YJ028, *S. venezuelae* 28-SET152, *S. venezuelae* 28-ACC152, *S. venezuelae* 28-pASA152, *S. venezuelae* 28-GG1, *S. venezuelae* 28-ACC152/GG1, and *S. venezuelae* 28-ASA152/GG1, after being cultured at 28 ºC for 96 h in SCM medium. The extract was analyzed by HPLC, LC-ESI/MS and GC/MS. Flaviolin was quantified by spectrophotometry on the basis of the molecular extinction coefficient (4.12) at 262 nm. The quantity was averaged from 4 different extractions.

5.2.5 Extraction of intracellular acetyl-CoA and malonyl-CoA

We speculated that the enhanced production of flaviolin in *S. venezuelae* 28-ASA152/GG1 and *S. venezuelae* 28-ACC152/GG1 was due to an increased pool of malonyl-CoA. To confirm this hypothesis, we extracted and quantified intracellular acetyl-CoA and malonyl-CoA from these strains as described in materials and methods. The acetyl-CoA and malonyl-CoA isolated from these strains were analyzed by HPLC (**Fig. 5-5**) and quantification was performed by calibration curve of the corresponding standard. **Table 5-1** shows a quantitative comparison of the acetyl-CoA and malonyl-CoA concentrations among different *S. venezuelae* YJ028 strains during batch cultivation. The intracellular concentrations of acetyl-CoA were similar to those in *S. venezuelae* YJ028 and *S. venezuelae* 28-SET152. *S. venezuelae* 28-GG1 had the lowest amount of acetyl-CoA among these strains. While quantifying malonyl-CoA, we observed that *S. venezuelae* 28-ACC152, *S. venezuelae* 28-pASA152, *S. venezuelae* 28-ACC152/GG1 and *S. venezuelae* 28-ASA152/GG1 produced significantly higher amounts of malonyl-CoA than that by *S. venezuelae* YJ028, *S. venezuelae* 28- SET152 or *S. venezuelae* 28-GG1.

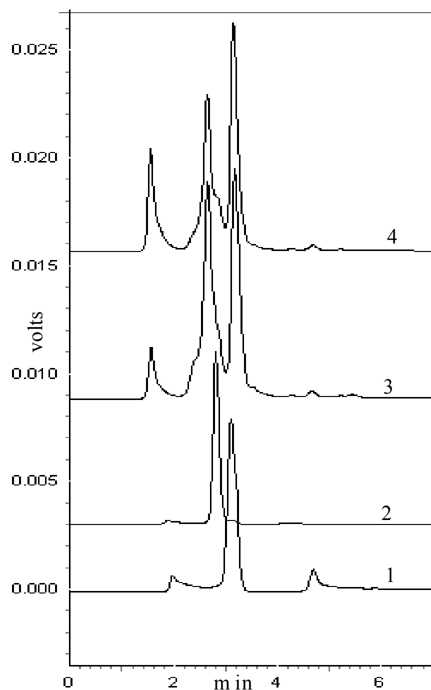


Figure 5-5. HPLC chromatogram of acyl-CoA isolated from *S. venezuelae* 28- ACC152/GG1 and *S. venezuelae* 28-ASA152/GG1. (1) Acetyl-CoA standard, (2) Malonyl-CoA standard, (3) Acyl-CoA isolated from *S. venezuelae* 28-ACC152/GG1 and (4) Acyl-CoA isolated from *S. venezuelae* 28-ASA152/GG1.

Table 5-1. Acyl-CoAs extracted from *S. venezuelae* YJ028 and the mutant

Culture Time	Strains	Acetyl-CoA	Malonyl-CoA
2 days	*S. venezuelae* YJ028	5.23 ±0.30	0.002±0.002
	S. venezuelae 28-SET152	5.27±0.30	0.004±0.003
	S. venezuelae 28-ACC152	5.33±0.42	5.39±0.32
	S. venezuelae 28-ASA152	5.45±.44	5.62±0.39
	S. venezuelae 28-GG1	2.35 ±0.36	0.003±0.001
	S. venezuelae 28-ACC152/GG1	3.44 ±0.33	4.61±0.26
	S. venezuelae 28-ASA152/GG1	5.23±0.40	5.62±0.33
4 days	*S. venezuelae* YJ028	11.28±0.24	1.40±0.02
	S. venezuelae 28-SET152	12.33±0.31	1.97±0.03
	S. venezuelae 28-ACC152	23.10±0.22	13.05±0.24
	S. venezuelae 28-ASA152	38.53±0.13	17.91±0.33
	S. venezuelae 28-GG1	10.25±0.41	1.53±0.33
	S. venezuelae 28-ACC152/GG1	21.63±0.26	16.92±0.33
	S. venezuelae 28-ASA152/GG1	35.26±0.19	23.06±0.40

Values are represented as µmoles/g dry cell weight

5.3 Discussion

We engineered *S. venezuelae* YJ028 so as to increase the precursor pool to be directed towards various polyketides. Since *S. venezuelae* requires a short culture period (3–4 days) for metabolite production, is amenable to genetic manipulation and has high transformation efficiency compared to other *Streptomyces* species, we chose to develop *S. venezuelae* as a heterologous host for polyketide production. Malonyl-CoA and methylmalonyl-CoA are the most common chain extender units for the biosynthesis of polyketide antibiotics. Biosynthesis of malonyl-CoA occurs in most species through ATP-dependent carboxylation of acetyl-CoA by an ACC. The reaction catalyzed by this enzyme is a two-step process that involves ATP-dependent formation of carboxybiotin, followed by transfer of the carboxyl moiety to acetyl-CoA (**Scheme 5-1**) (Davis *et al*., 2000). Therefore, diverting carbon flux from acetyl-CoA away from citrate and directing it toward malonyl-CoA should lead to higher antibiotic production.

Methylmalonyl-CoA (mm-CoA) can be synthesized in two ways: first, through the action of PCC; and, second, in the reverse reaction of the mm-CoA mutase (MCM) and epimerase pathway from succinyl-CoA. Propionyl-CoA is a central metabolite in the degradation of the amino acids, L-Met, L-Ile and L-Val. It is also the end product in the degradation of uneven fatty acids by β-oxidation. According to biochemistry textbooks, propionyl-CoA is carboxylated to (2S)-methylmalonyl-CoA by the biotin-dependent PCC (**Scheme 5-2**). As we know, many polyketides require methylmalonyl-CoA as an extender

unit for the incorporation of C_3-units, diverting carbon flux from propionyl-CoA towards methylmalonyl-CoA should also lead to the higher production of respective polyketide compound.

For this, a gene complex for ACC and PCC were separately expressed in *S. venezuelae* YJ028 to enhance the carbon flux through acetyl-CoA to malonyl-CoA and propionyl-CoA to methylmalonyl-CoA respectively. Moreover, signal molecule, methyl group donor *metK1-sp* and global regulatory gene *afsR-sp* were also integrated into the chromosomal DNA of *S. venezuelae* YJ028 to enhance the regulation.

To study whether the newly developed *S. venezuelae* YJ028 hosts efficiently produce heterologous polyketides or not, we expressed type III polyketide synthase gene, s*p-rppA*, which encodes 1,3,6,8-tetrahydroxynaphthalene synthase (THNS). Sp-RppA catalyzes the condensation of five molecules of malonyl-CoA to form THN, which readily oxidizes to give 2,5,7- trihydroxy-1,4-naphthoquinone (flaviolin). The isolation and analysis of flaviolin produced by *S. venezuelae* YJ028, *S. venezuelae* 28-SET152, *S. venezuelae* 28-ACC152, *S. venezuelae* 28-pASA152, *S. venezuelae* 28-GG1, *S. venezuelae* 28-ACC152/GG1 and *S. venezuelae* 28-ASA152/GG1 showed that the newly developed *S. venezuelae* 28-ACC152 and 28-ASA152 are more efficient heterologous hosts than the parent *S. venezuelae* YJ028. The enhanced production of flaviolin in *S. venezuelae* 28-ASA152/GG1 and *S. venezuelae* 28-ACC152/GG1 is due to increased pool of malonyl-CoA as revealed by comparative quantification of intracellular acetyl-CoA and malonyl-CoA from these strains during their batch cultivation. These analyses revealed that newly-developed hosts, *S. venezuelae* 28-

ACC152, *S. venezuelae* 28-pASA152, *S. venezuelae* 28-ACC152/GG1 and *S. venezuelae* 28-ASA152/GG1 produced significantly higher amounts of malonyl-CoA compared to *S. venezuelae* YJ028, *S. venezuelae* 28-SET152 or *S. venezuelae* 28-GG1.

On the basis of these results, we concluded that the increased production of flaviolin is due to the increased pool of malonyl-CoA in the respective strains. Similarly, we can use *S. venezuelae* 28-PCC152 and *S.venezuelae* 28-PSA152 that are capable of producing increased amount of methylmalonyl-CoA pool to synthesize important polyketides that need methylmalonyl CoA precursors for their biosynthesis.

From this study, it is evident that the *S. venezuelae* YJ028 strain expressing ACC, *metK1-sp* and *afsR-sp* is a more efficient host for greater heterologous production of polyketides than the parent host, *S. venezuelae* YJ028. Thus, through metabolic engineering, we have developed *S. venezuelae* YJ028 as an efficient heterologous host for enhanced heterologous production of various polyketides via modification of precursor supplies in *S. venezuelae* YJ028.

Chapter VI

Activation of biosynthesis of actinorhodin and red compounds in *Streptomyces chromofuscus* ATCC 49982

6.1 Background

In modern agriculture, application of herbicide is essential to maintain high productivity since crop losses from weeds worldwide average 10% annually (Mallik, 2001). Eventhough synthetic chemicals have played a significant and unparalleled role in enhancing crop yields by suppressing pests, their heavy application has resulted in both ecological and medical problems. Increased awareness of the environmental and health problems caused by continious use of synthetic herbicides has aroused great interest in biodegradable, selective and environmentally friendly herbicides. The utilization of microorganisms and their metabolic by-products has become a thrust area of research among the scientists searching for natural product alternatives to conventional herbicides.

Actinomycetes are the major source of several herbicides and other bioactive compounds. Herbicidal activity of the compounds produced by the Actinomycetes came into the limelight with the discovery of bialaphos (**Fig. 6-1**) from *S. hygroscopicus* and *S. viridochromogenes* in 1973. A large number of nucleoside compounds produced by *Streptomyces* species are herbicidal in nature.

One of the well-known commercial herbicide of microbial origin is herboxidiene (also known as TAN-1609) which is isolated from *Streptomyces* sp. A 7847 by the workers of Monsanto. Herboxidiene (**Fig. 6-1**) is a polyketide natural product that is structurally interested by the tetrahydropyran moiety and the side chain including a conjugated diene. It was found to control (>90%) several important biannual weeds at relatively low application

rates (<250 g/hectare) without damaging wheat (Wideman *et al*., 1991). Early synthetic work indicated that the epoxide and the C-18 hydroxyl groups are important for the activity of herboxidiene.

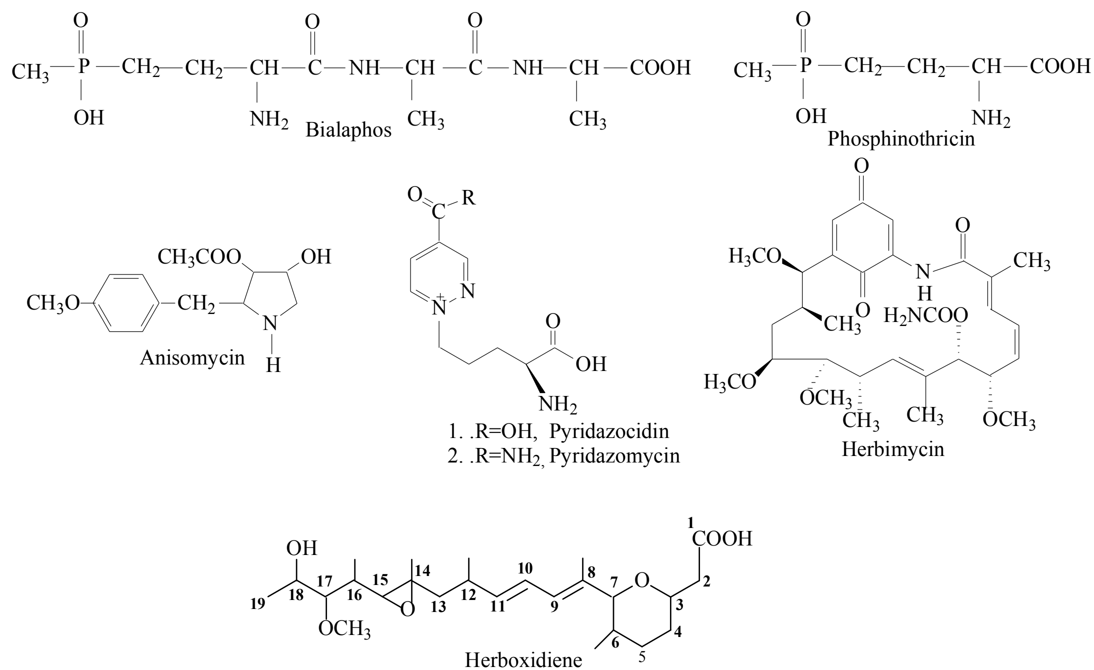


Figure 6-1. Herbicidal polyketide compounds from different *Streptomyces* species.

In 2002, herboxidiene was isolated from a culture broth of *Streptomyces sp*. along with five novel structurally related compounds, GEX1Q1, GEX1Q2, GEX1Q3, GEX1Q4 and GEX1Q5 (Sakai *et al*., 2002). The same group reported that herboxidiene and all related compounds show antitumor activity against human tumor cell lines. Furthermore,

herboxidiene up-regulates the gene expression of low density lipoprotein receptors (Koguchi *et al*., 1997).

The interesting biological profile of this natural product has prompted various attempts to produce it and its analogues by chemical synthesis. Simplified aromatic hybrids of the herboxidiene were synthesized and it was found that one of those hybrids showed significant herbicidal activity against broad-leaved weeds in post-emergent application (Edmunds *et al*., 2000). It is well known that most important aspect of natural product biosynthesis is the generation of the diverse chemical structures, which are difficult to synthesize by synthetic approaches. Therefore, genetic engineering represents a potent approach towards chemical structural diversification and analog production.

The potent herbicidal activity of the molecule, as well as the discoveries that it up-regulates gene expression of low density lipoprotein receptors, and shows anti-tumor activity, suggest this natural product would be an ideal candidate for analogue biosynthesis. Moreover, the presence of two interesting features in herboxidiene: the *cis*-substituted tetrahydropyran acetic acid core and the *E*,*E*-diene moiety situated between carbons 7–12 make it an appropriate candidate for biosynthetic engineering to generate novel analogues.

Thus, in this study, in pursuit of mass production of eco-friendly herbicide and also to generate novel analogues of herboxidiene, we carried out the metabolic engineering of *S. chromofuscus* ATCC 49982. One of the metabolic engineering processes where a particular metabolite can be overproduced is to alter the metabolic flux distribution of its different precursors since the availability of biosynthetic precursors is a key factor determining the

productivity of secondary metabolites. For this, a gene complex for acetyl-CoA carboxylase (*accA2+accB+accE*) and propionyl-CoA carboxylase (*prpE+accA1+pccBE*) along with methyl group donor *metK1-sp* (Zhao *et al*., 2006) and global regulatory gene *afsR-s*p (Parajuli *et al*., 2005) were separately expressed in *S. chromofuscus* ATCC 49982. ACC catalyses the biosynthesis of malonyl coenzyme A through ATP-dependent carboxylation of acetyl-CoA (Rodriguez *et al*., 2001; Tong, 2005). Similarly, PCC catalyses the biosynthesis of methylmalonyl coenzyme A by carboxylation of propionyl-CoA (Rodriguez *et al*., 1999).

6.2 Results

6.2.1 Optimization of isolation of herboxidiene from *S. chromofuscus*

Herboxidiene is a polyketide compound isolated from *S. chromofuscus* A7847, which is active against several important weed species. We purchased *Streptomyces sp*. A 7847 (*S. chromofuscus* ATCC 49982) from American Type Culture Collection (ATCC). The morphology of wild type *S. chromofusus* on ISP2 agar plate is shown in **Fig. 6-2**. We optimized the isolation of herboxidiene in our lab condition. We have slightly modified the isolation method and HPLC condition of herboxidiene as described in materials and methods. Herboxidiene isolated from *S. chromofusus* wild type was purified and analyzed by HPLC at 236 nm (**Fig. 6-3**) and the peak corresponding to herboxidiene was confirmed by LC/MS analysis (**Fig. 6-4**).

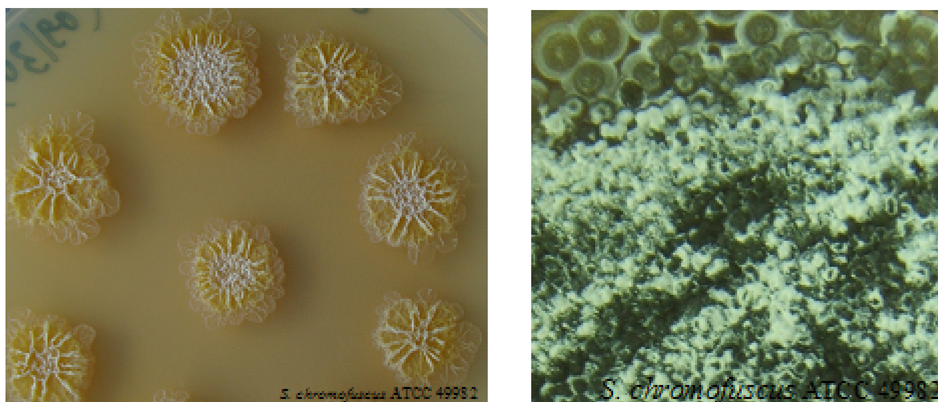


Figure 6-2. Morphology of *S. chromofuscus* ATCC 49982 on ISP2 agar plate after 5 days and 8 days of incubation at 28ºC.

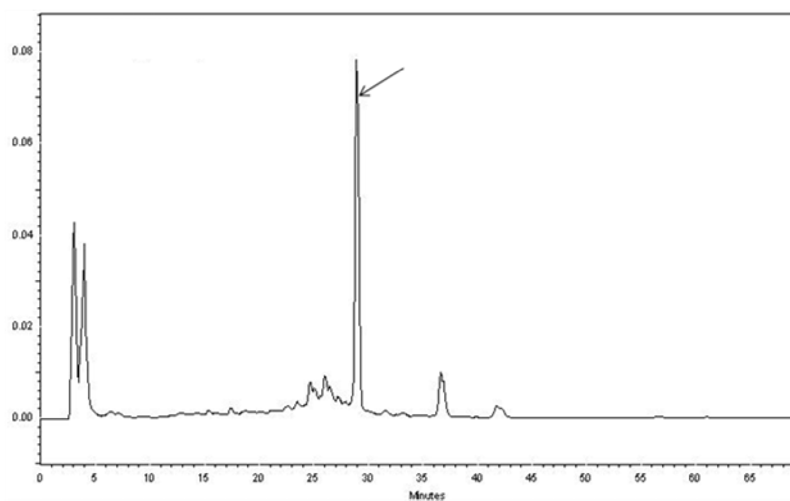


Figure 6-3. HPLC chromatogram of herboxidiene isolated from *S. chromofuscus* wild type strain. The peak corresponding to herboxidiene is indicated by arrow head.

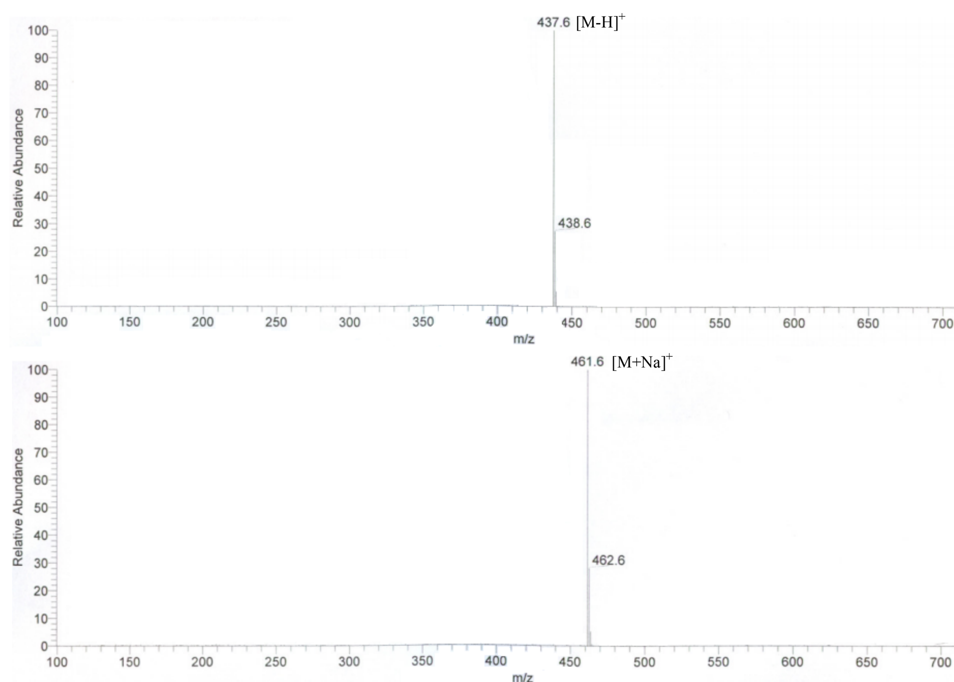


Figure 6-4. LC/MS spectrum of herboxidine in negative and positive mode. Molecular mass of herboxidiene [M] = 438.

6.2.2 Effect of heterologous expression of ACC and PCC on morphology of *S. chromofuscus*

As a result of the increasing environmental and health-related problems caused by the synthetic agrochemicals, non-hazardous alternatives are being sought. Herbicides produced by the *Streptomyces* strains have been proved to be natural and eco-friendly herbicides. Seeking biodegradable and environmentally friendly herbicides, we intended to enhance the production of herboxidiene through metabolic engineering of *S. chromofuscus* ATCC 49982 so as to increase different precursor pool. Since the herboxidiene was

proposed to be biosynthesized by condensation of acetyl-CoA and propionyl-CoA, the recombinant plasmids pACC152, pPCC152, pASA152, and pPSA152, harboring ACC, PCC and also ACC and PCC genes along with *afsR-sp* and *metK1-sp* were expressed separately into *S. chromofuscus* resulting *S. chromofuscus* ACC152, *S. chromofuscus* PCC152, *S. chromofuscus* ASA152, and *S. chromofuscus* PSA152 respectively following usual protoplast transformation method. *S. chromofuscus* SET152 harboring pSET152 was used as a control in the study.

Surprisingly, we observed that the morphology of all transformants including *S. chromofuscus* SET152 was drastically different from that of *S. chromofuscus* parent strain in R2YE liquid and agar plates. Morphology of all strains was compared in herboxidiene production liquid media as well as in ISP2 liquid and agar plates. In all of these media, the morphology of transformants was observed to be completely different from that of parent strain. All strains appear as deep blue colonies in ISP2 agar plates (**Fig. 6-5**).

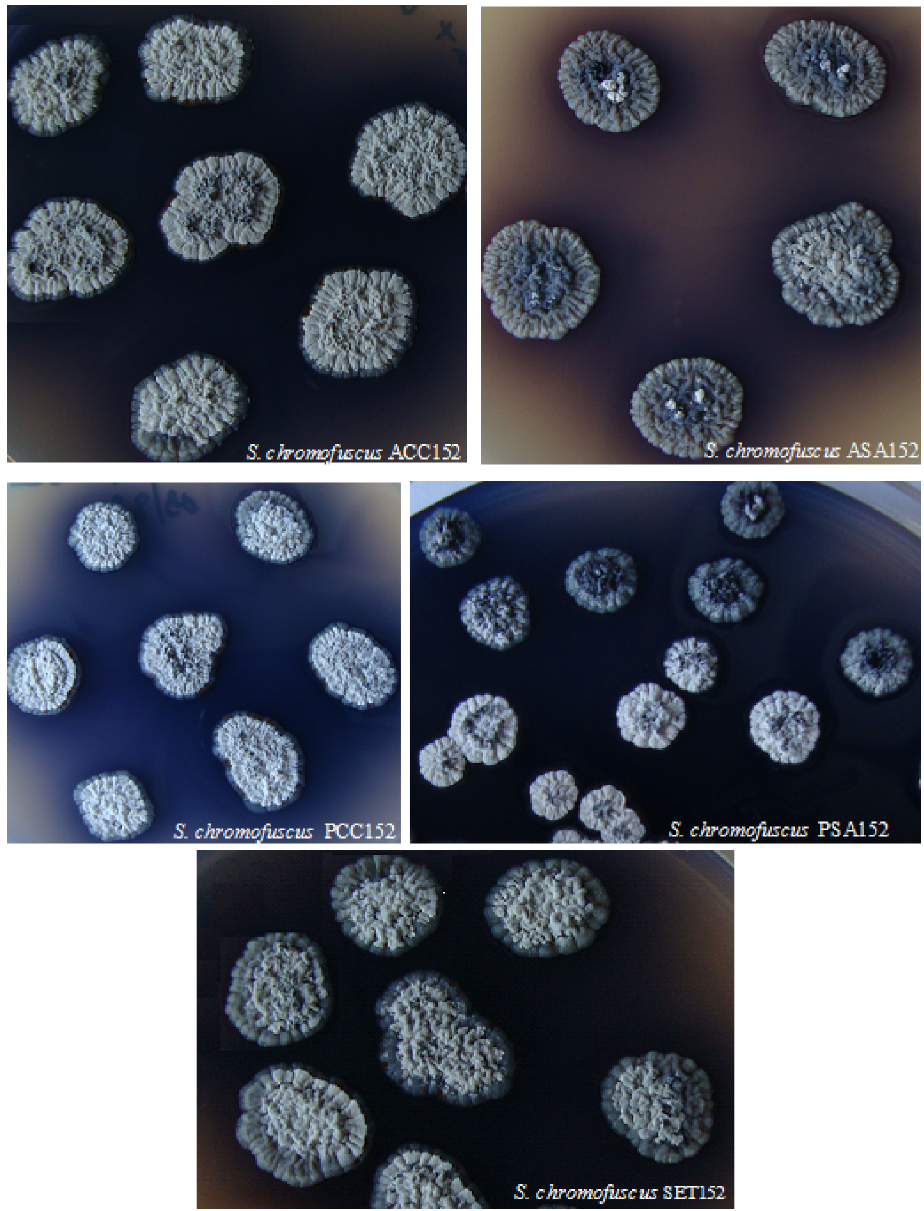


Figure 6-5. Morphology of *S. chromofuscus* transformants on ISP2 agar plates.

6.2.3 Activation of actinorhodin and red compounds biosynthesis in *S. chromofuscus*

Irrespective of the genes expressed, all *S. chromofuscus* mutant strains including *S. chromofuscus* SET152 produced blue and red pigmented compounds. Although change in morphology was observed in R2YE and herboxidiene production media, ISP2 solid media was used for isolation of pigmented compounds (actinorhodin and undecylprodigiosin), since a blue diffusible pigment production was seen higher on ISP2 agar than in R2YE and herboxidiene production media. Moreover, among all *S. chromofuscus* mutant strains, *S. chromofuscus* PCC152 and *S. chromofuscus* PSA152 were observed to produce higher amount of blue diffusible pigments. Thus, the pigmented compounds were first isolated from *S. chromofuscus* PCC152 and *S. chromofuscus* PSA152. It was found that both strains produced mixture of compounds that comprised of blue and two red colored compounds (**Fig. 6-6**). It appeared as reddish blue spot in TLC assay (**Fig. 6-6**) and turned blue on fuming with ammonia. The blue pigmented compound was highly soluble in water and when the pH of blue compound was changed to pH 2 to 3 with 1 M HCl, the blue color changed into pink (**Fig. 6-7**). λ_{max} of blue pigmented compound was found to be 633 nm as shown by UV spectrophotometer (**Fig. 6-8**). Red compound A was insoluble in water, moderately soluble in methanol, ethanol, and hexane and readily soluble in diethyl ether, ethylacetate and DMSO. Red compound A was orange-yellow at basic pH and pinkish-red at acidic pH. λ_{max} of red pigmented compound A was found to be 533 nm in acidic condition and 468 nm in basic condition (**Fig. 6-9**). The red compound was further analyzed by MALDI-TOF-

TOF-MS (**Fig. 6-10**) and ^{1}H-NMR (**Fig. 6-11**). It was further confirmed by ^{13}C-NMR, HMBC, HMQC, COSY, ROESY, NOESY and DOSY analyses. Thus, from all above experimental data we came to conclusion that the blue pigmented compound is pH indicator and antibiotic compound, actinorhodin and red compound A is immunosuppressive drug, undecylprodigiosin (**Fig. 6-12**).

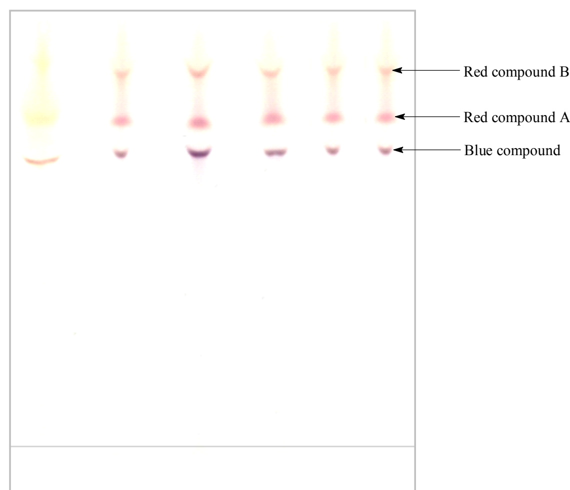


Figure 6-6. TLC assay of pigmented compounds isolated from *S. chromofuscus* wild type and transformants. 1, compounds from *S. chromofuscus* wild type; 2, compounds from *S. chromofuscus* PCC152; 3, compounds from *S. chromofuscus* PSA152; 4, compounds from *S. chromofuscus* SET152; 5, compounds from *S. chromofuscus* ASA152; 6, compounds from *S. chromofuscus* ACC152.

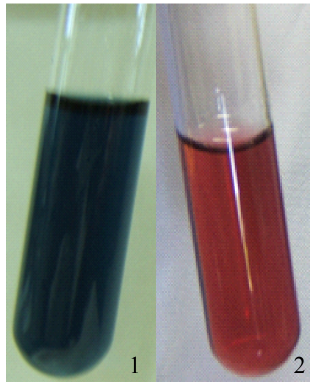


Figure 6-7. Blue pigmented compound at basic pH (1) and acidic pH (2) isolated from *S. chromofuscus* transformants.

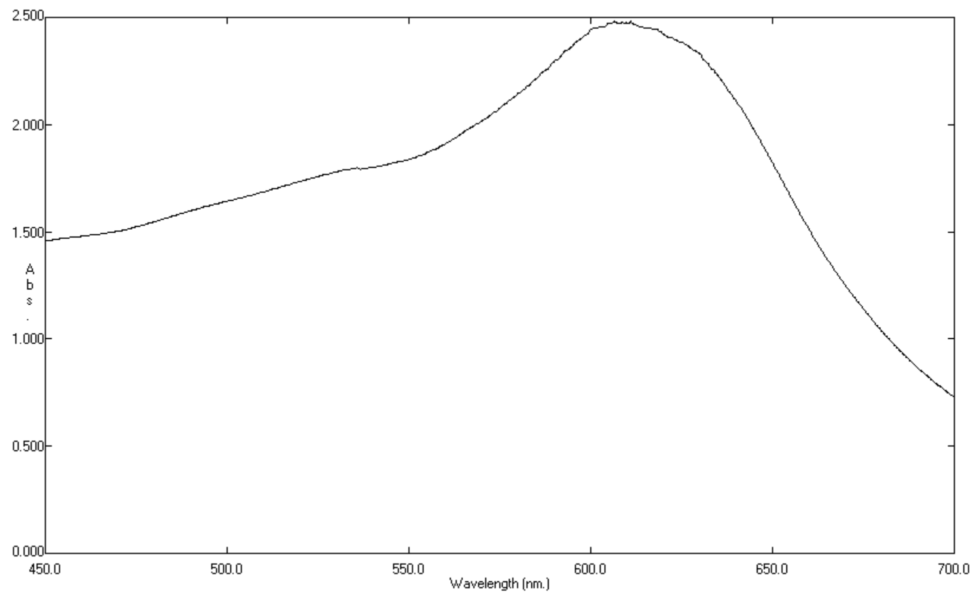


Figure 6-8. λ_{max} of blue compound at basic pH.

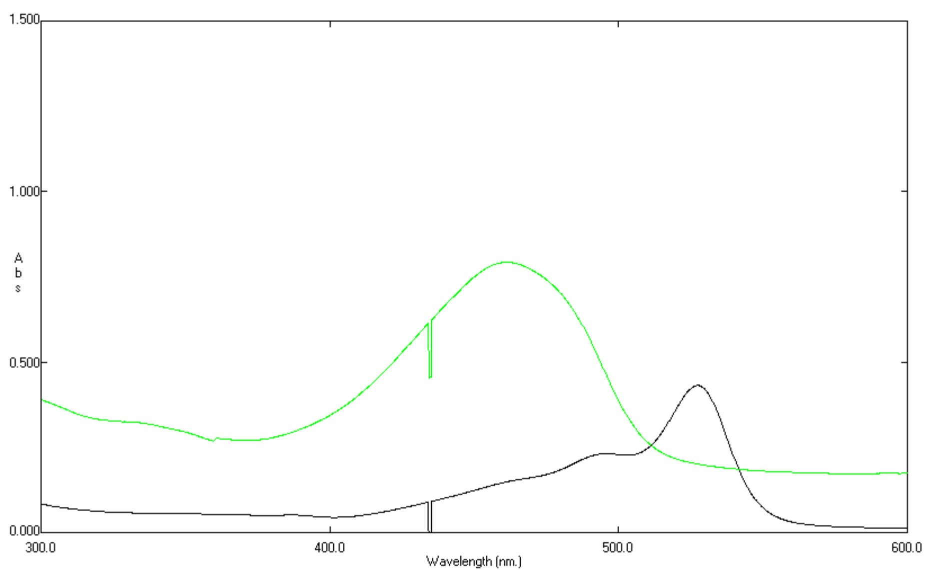


Figure 6-9. λ_{max} of red compound A at acidic and basic conditions.

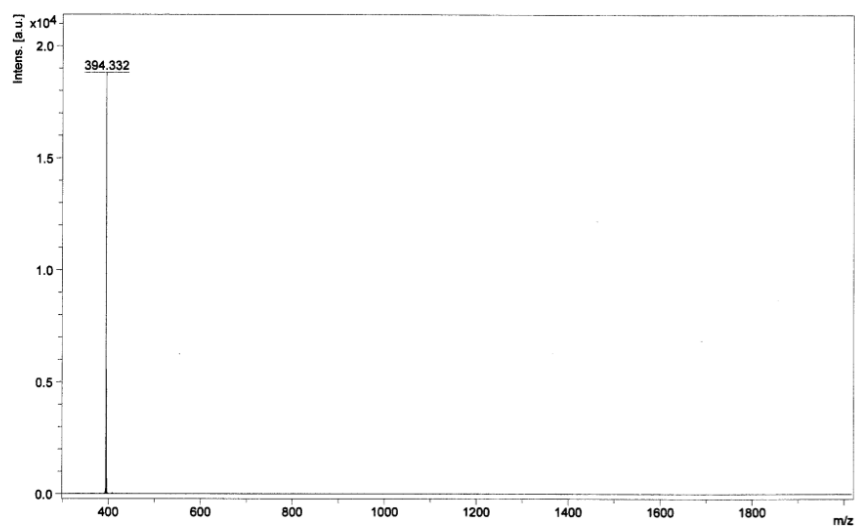


Figure 6-10. MALDI-TOF-TOF-MS analysis of red compound A.

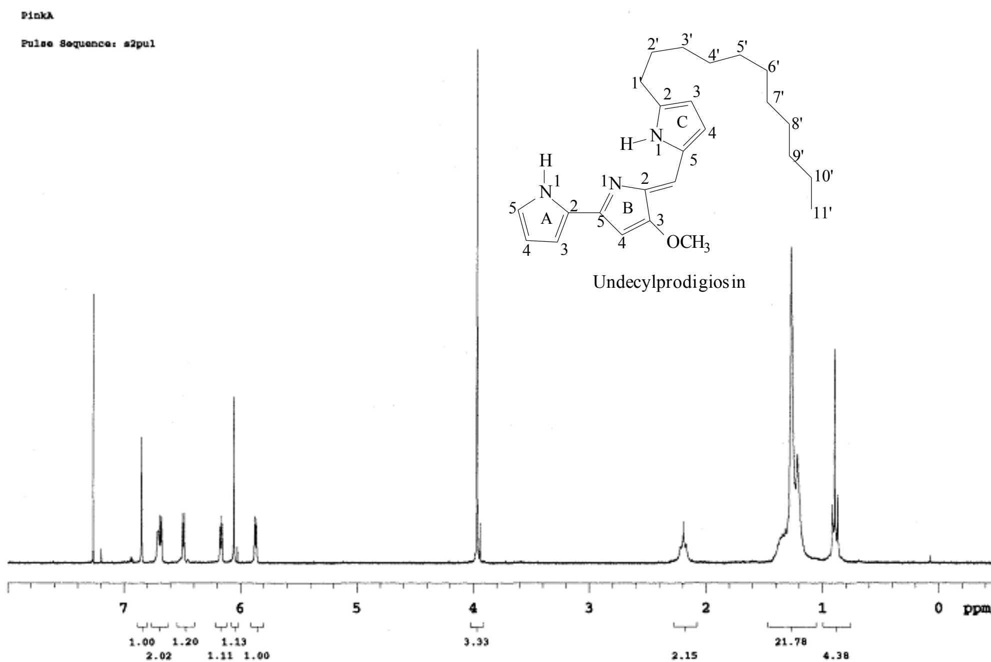


Figure 6-11. ^{1}H-NMR of Red compound A.

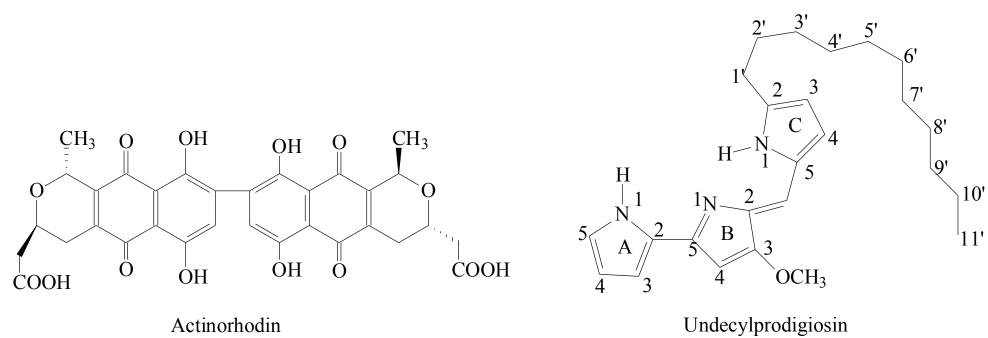


Figure 6-12. Structures of actinorhodin and undecylprodigiosin.

6.2.4 Quantitative analysis of pigmented compounds

To analyze whether the compounds produced by all these *S. chromofuscus* mutant strains are same or not, we carried out isolation and purification of the compounds from all strains under similar conditions as described in materials and methods. From these analyses, we found that all these strains produced same blue and red pigmented compound. The blue and red pigmented compounds produced by these strains were quantified after purification and compared with that produced by *S. coelicolor*. The amount of purified blue and red compounds is shown in **Fig. 6-13** and **Fig. 6-14**.

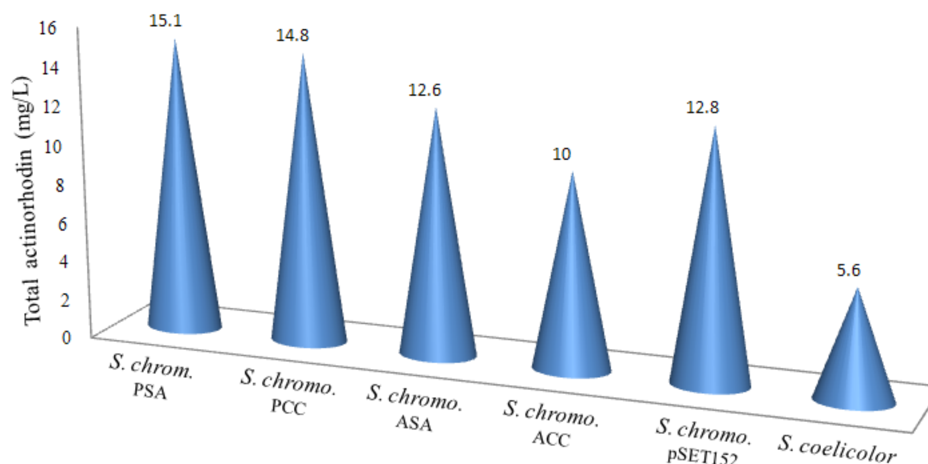


Figure 6-13. Quantification and comparison of total amount of purified blue pigmented compound produced by different *S. chromofuscus* transformants and *S. coelicolor* in ISP2 agar plates.

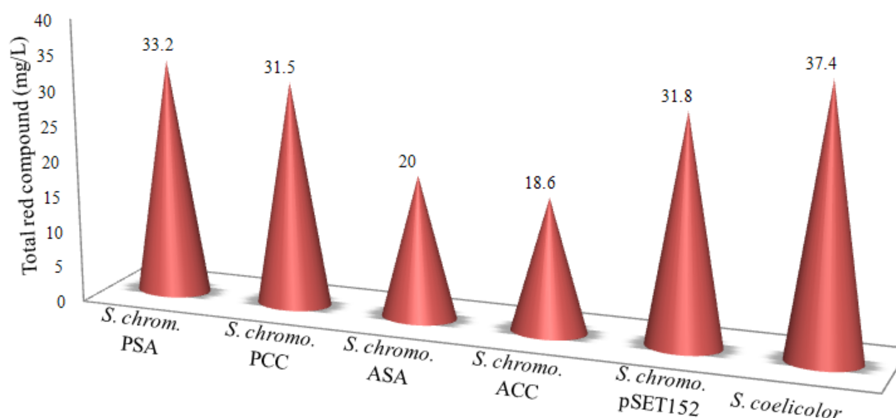


Figure 6-14. Quantification and comparison of total amount of purified red pigmented compound produced by different *S. chromofuscus* transformants and *S. coelicolor* in ISP2 agar plates.

6.3 Discussion

Members of the genus *Streptomyces* produce natural product antibiotics most of which are used clinically today. The activation of antibiotic production, often coupled to morphological development, involves many different pathways in the same organism. Multiple and coordinated regulatory mechanisms controlling antibiotic biosynthesis are still poorly understood. *S. coelicolor* A3 (2), the best genetically studied *Streptomyces*, produces four biochemically and genetically distinct antibiotics: actinorhodin (Act), undecylprodigiosin (Red), methylenomycin and calcium-dependent antibiotic (CDA). The gene clusters responsible for the production of Act, Red, methylenomycin and CDA have been cloned and characterized (Bystrykh *et al*., 1996).

S. chromofuscus ATCC 49982 which is the producer of herbicide, herboxidiene, normally produces no or only negligible amount of actinorhodin and undecylprodigiosin throughout the whole-cell cycle. However, when we attempted to enhance herboxidiene from *S. chromofuscus* by overexpression of ACC and PCC separately, surprisingly in every case, even in case of *S. chromofuscus* SET152 harboring only the vector, the strains showed dramatic change in morphology and the secondary metabolites production. The morphology of these strains are similar to that of *S. coelicolor* A3 (2). All of these *S. chromofuscus* mutant strains produced high amount of blue diffusible pigments as shown in **Fig. 6-4**. When the pigmented compounds were isolated and analyzed, we found that all these strains produced blue pigment antibiotic, actinorhodin and red colored compound, undecylprodigiosin. Actinorhodin is a blue/red (pH-dependent) secondary metabolite belonging to the aromatic polyketides. Acetyl-CoA is the only carbon precursor for its synthesis (Fernandez-Moreno *et al*., 1994; Bystrykh *et al*., 1996). Undecylprodigiosin (Red) is a bacterial bioactive metabolite produced by *Streptomyces* and *Serratia*. Red is the first described member of a family of related compounds showing immunosuppressive activity and is biosynthesized from acetyl-CoA and pyrrol-2-carboxylic acid.

To date, several studies have been reported the activation of production of actinorhodin and undecylprodigiosin in *S. coelicolor* A3 (2) or its close relative *S. lividans*. The *S. lividans* wild type does not usually produce actinorhodin when grown in liquid culture even though it carries the entire cluster for actinorhodin production, it usually remains unexpressed. Introduction of a multicopy plasmid carrying *afsR* or *abaA* or an

activator gene from *S. lividans* activates actinorhodin production in *S. lividans* (Horinouchi *et al*., 1989; Fernandez-Moreno *et al*., 1992), while mutational disruption of the *absA* locus results in precocious hyperproduction of actinorhodin and undecylprodigiosin in *S. coelicolor* A3(2) (Brian *et al*., 1996). It was reported that introduction of the *str* mutation, which confers resistance to streptomycin, could activate actinorhodin production in *S. lividans*. Earlier studies showed that a 4.3 kb gene fragment from *S. griseus* ATCC 10137 under the control of *ermE** promoter stimulated the production of actinorhodin and undecylprodigiosin in *S. lividans* TK24 which is the non producer of these compounds in normal conditions. The mechanism that stimulates Act and Red biosynthesis in *S. lividans* is still not clear.

Although Act and Red compounds are biosynthesized from acetyl-CoA precursors, since *S. chromofuscus* SET152 also produced the same compounds, the activation of biosynthesis of these compounds in *S. chromofuscus* strains was not only due to enhanced pool of precursors. It is possible that a threshold concentration of the activator protein is needed for activating the antibiotic biosynthesis genes. The low level of pathway regulatory ActII-ORF4 or RedD protein expressed in the wild-type *S. chromofuscus* strain apparently was not enough to activate Act and Red biosynthesis. Thus, we speculated that when ACC and/or PCC was expressed under the control of strong *ermE** promoter the activation of biosynthesis of actinorhodin and red compounds in *S. chromofuscus* mutant strains might be occured by indirectly or directly upregulating the expression of the antibiotic biosynthetic pathway-specific regulatory proteins. Since, biosynthesis of Act and Red was also occurred

in *S. chromofuscus* SET152 also, it is possible that *ermE** promoter upregulated the expression of the antibiotic biosynthetic pathway-specific regulatory proteins by unknown mechanism.

Thus in this study we got the unexpected but fruitful results. The biosynthesis of important compounds such as actinorhodin and undecylprodigiosin was activated in the non producer strain, *S. chromofuscus* ATCC 49982 and the quantity of these compounds were found to be similar to that produced by the natural producer *S. coelicolor*.

Chapter VII

Metabolic engineering of *Nocardia* sp. CS682 for enhanced production of nargenicin A

7.1 Background

Natural products are an important source of drugs that encompass antibiotics, anti-infectives and anticancer drugs. It is anticipated that many compounds with novel or enhanced therapeutic effects can be developed from compounds present in natural product libraries.

Nocardia species are pathogens of the human host. Although most of them have been isolated from clinical specimens, some were isolated from soils and reported to be producers of various biologically active compounds such as antimicrobial, cytotoxic, immunosuppressive and antifungal agents (Blaine *et al*., 1994). They have complex cell walls containing covalently associated peptidoglycans, arabinogalactans and mycolic acids and are rich in complex polysaccharides (lipoarabinomannans, glucans, and mannans), long-chain multimethyl-branched waxes and phospholipids. They cannot easily be disrupted by standard chaotropic or detergent solutions used for the rapid lysis of other bacteria (Blaine *et al*., 1994). Thus, their isolation, identification and manipulation have been limited by available microbiological methods.

Nargenicin A_1 (**Fig. 7-1**) is a member of a novel class of saturated alicyclic polyketides containing a characteristic *cis*-fused octalin ring system. Structurally, it is closely related to antibiotic nodusmicin (Whaley *et al*., 1980). A similar, trans- fused, octalin nucleus is found in the Actinomycete metabolites chlorothricin , kijanimicin , and tetrocarcin A as well as in the fungal antibiotic ilicicolin H. Nargenicin A_1 was first isolated as

antibioticum CP-47,444 from cultures of *Nocardia argentinensis* in 1977 (Celmer *et al*., 1980) and was proved to be pyrrole-2-carboxylate ester of nodusmicin. Recently, we isolated and purified nargenicin A_1 from the novel Actinomycete strain CS682 KCTC 11297BP (*Nocardia* sp. CS682), from soil in Jeonnam, Korea (Sohng *et al*., 2008). Nargenicin A_1 has strong antibacterial activity against methicilin-resistant *Staphylococcus aureus* (Seung *et al*., 2009). Activity of nargenicin A1 against *Staphylococcus aureus* is comparable to that of erythromycin (**Table 7-1**). Besides, earlier reports presented the evidences that nargenicin inhibited cell proliferation and induced HL-60 cell differentiation when administered in combination with 1,25-(OH)2D3 or ATRA and enhanced leukemia cell differentiation via PKCbeta1/MAPK pathways. Thus, nargenicin has the ability to induce differentiation and suggest that it may be useful for the treatment of neoplastic diseases (Seung *et al*., 2009).

Number of multidrug-resistant gram-positive bacterial pathogens exists, and their rising numbers are a major concern. The occurence of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) reveals the seriousness of this concern. Thus, either the development of new antimicrobial agents or increment of existing drugs is crucial to reduce the future clinical impact of resistant pathogens. Finding novel antibiotics active against such drug-resistant gram-positive pathogens, however, is difficult. Structural modification of the known natural products is often essential in drug development, e.g., for improvements in efficacy and pharmacokinetics. The manipulation of genes which encode enzymes of the biosynthetic pathways represents a promising approach for

introducing structural changes. The functional analysis of biosynthetic genes is, however, a prerequisite for such approaches.

In the current study, knowing the therapeutic importance of nargenicin A1, we prompted to enhance the production of nargenicin by metabolic engineering and feeding experiments. In an attempt to enhance the production of nargenicin, we first expressed only S-adenosylmethionine synthetase (MetK1-sp) from *S. peucetius*. Then after, since studies have shown that nargenicin aglycon is derived from common precursors acetate and propionate, specifically 4 propionate and 5 acetate building blocks and the pyrrol moiety is derived from proline, we carried out expression of ACC together with proline feeding in *Nocardia* sp. CS682 to enhance production of a nargenicin A1. For this, besides the principal limitations presented by the complex cell wall as well as the lack of suitable protocols for the genetic manipulation of *Nocardia* species, we optimized the conditions for the transformation method and isolation of genomic DNA from *Nocardia* sp. CS682.

Table 7-1. Activity of nargenicin A_1 and erythromycin

Organism	**Antibiotics and MIC (µg/ml)**	
	Nargenicin A_1	Erythromycin
Staphylococcus aureus	0.1	0.1
*Staphylococcus aureus**	0.2	>200
Staphylococcus epidermis	0.8	0.1
*Staphylococcus epidermis***	0.8	>200
Neisseria sicca	25	1.6
Escherichia coli	2	1.6
Pasteurella multocida	25	0.4

*Multiple drug resistant strain,**Methicillin/erythromycin resistant strain

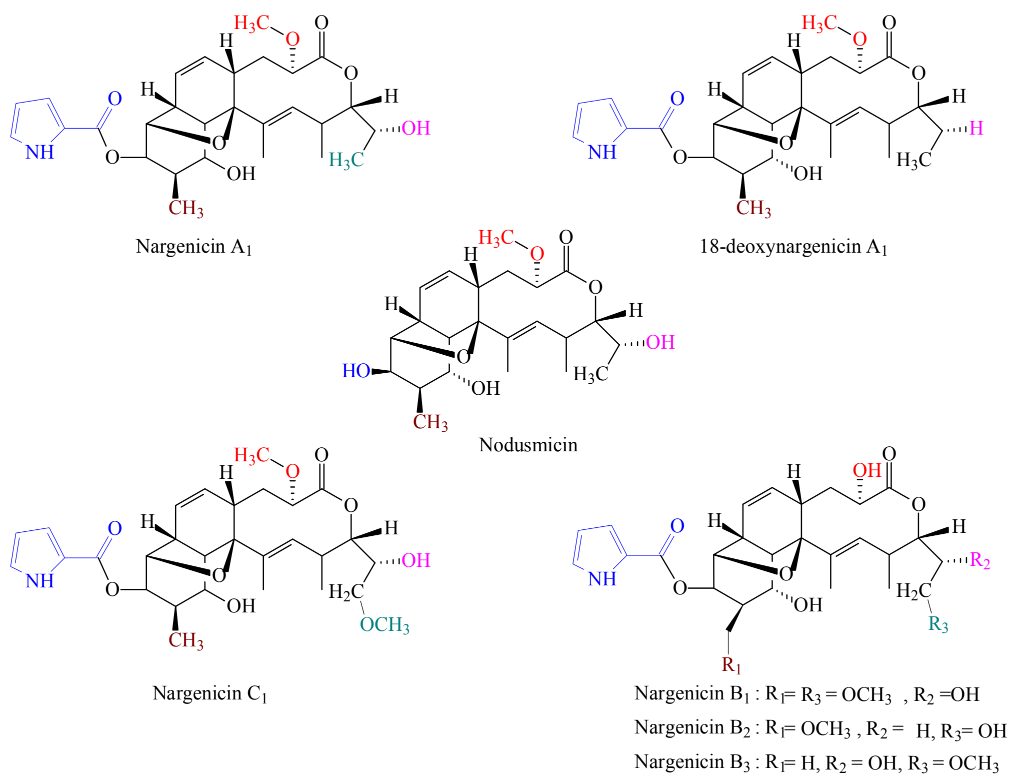


Figure 7-1. Nargenicin A_1 and its analogs.

7.2 Results

7.2.1 Expression of S-adenosylmethionine synthetase (MetK1-sp)

S-adenosylmethionine synthetase (MetK1-sp) was expressed in *Nocardia* sp. CS682 to study its effect on the production of nargenicin by cloning it into pNV18 as described in materials and methods. Similarly, for comparative study, *Nocardia* sp. CS682 was also transformed with only pNV18 (Chiba *et al*., 2007).

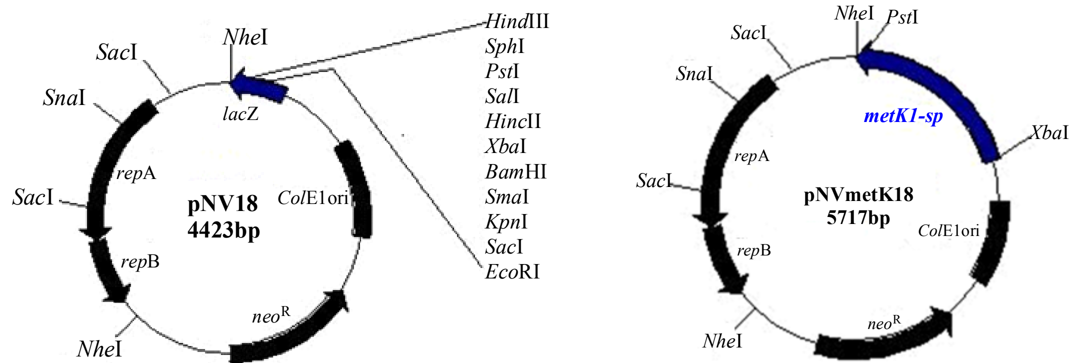


Figure 7-2. *Nocardia-E. coli* shuttle vector, pNV18 and recombinant plasmid pNVmetK18 harboring *metK1-sp.*

To compare the production of nargenicin by *Nocardia* sp. metK18 with that of *Nocardia* sp. CS682 and *Nocardia* sp. NV18*,* nargenicin was extracted from these strains under similar conditions and analyzed by HPLC (**Fig. 7-3**) and LC/MS analyses. To quantify the nargenicin, the calibration curve of nargenicin was used (Cho *et al*., 2009). From these experiments we found that the *Nocardia* sp. metK18 produces the increased amount of nargenicin as compared to wild type and *Nocardia* sp. NV18 (**Fig. 7-4**). Nargenicin was found to be enhanced by about 2.8 times in *Nocardia* sp. metK18 as compared to that of *Nocardia* sp. CS682 and *Nocardia* sp. NV18. The experiment was averaged from 4 different extractions.

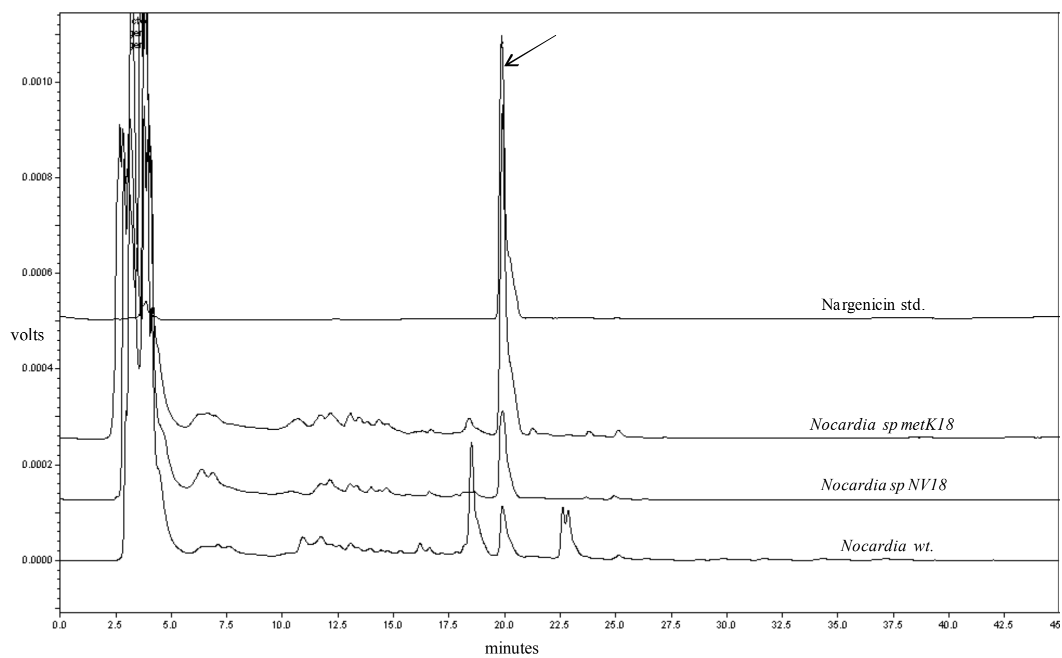


Figure 7-3. HPLC chromatogram of compounds isolated from *Nocardia* sp. CS682 and its transformants. The peak corresponding to nargenicin is indicated by an arrow head.

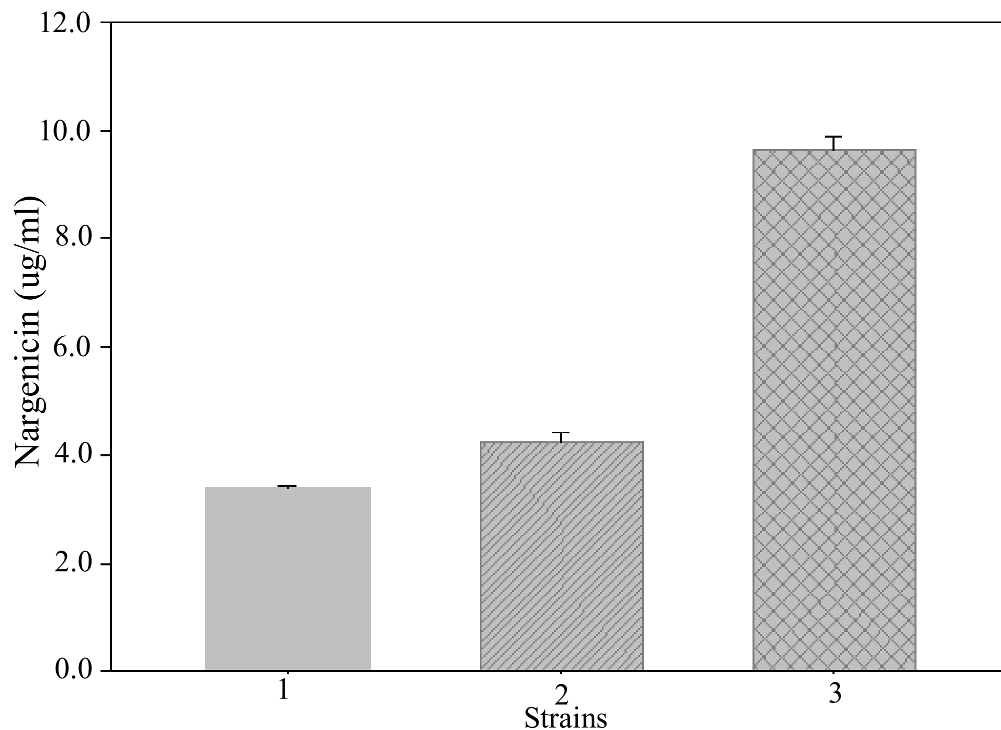


Figure 7-4. Comparison of nargenicin production from *Nocardia* sp. CS682 and its transformants. 1. Nargenicin produced by *Nocardia* wild type, 2. Nargenicin produced by *Nocardia* sp. NV18, 3. Nargenicin produced by *Nocardia* sp. metK18.
The experiment was averaged from 4 different extractions.

7.2.2 Expression of ACC in *Nocardia* sp. CS682

The positive results that were obtained by the expression of *metK1-sp* in *Nocardia* sp. CS682 raised our interest to enhance nargenicin production to higher extent. Since studies have shown that nargenicin aglycon is derived from common precursors, acetate and propionate, our next strategy was to express ACC in *Nocardia* sp. CS682.

Thus the ACC genes, *accA2* and *accBE*, (**Fig. 7-5**) from *S. coelicolor* were expressed as described in materials and methods and its effect on the production of nargenicin was studied by isolation and analyses of the compound from *Nocardia* sp. CS682, *Nocardia* sp. NV18 and *Nocardia* sp. ACC18 under similar conditions. From four different experiments we found that the production of nargenicin in *Nocardia* sp. ACC18 was increased by about 3.85 times than that produced by *Nocardia* sp. CS682, and *Nocardia* sp. NV18 (**Fig. 7-6**). Besides the increased production of nargenicin, every time when we isolated nargenicin from *Nocardia* sp. ACC18, we observed that a new peak corresponding to another compound was found to be increased much more than nargenicin as revealed by HPLC analyses (**Fig. 7-7**) and TLC assay (**Fig. 7-8**) with hexane: ethyl acetate as mobile phase.

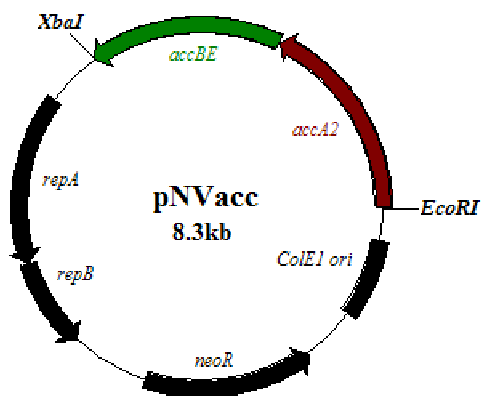


Figure 7-5. Recombinant plasmid harboring acetyl-CoA carboxylase genes from *S. coelicolor.*

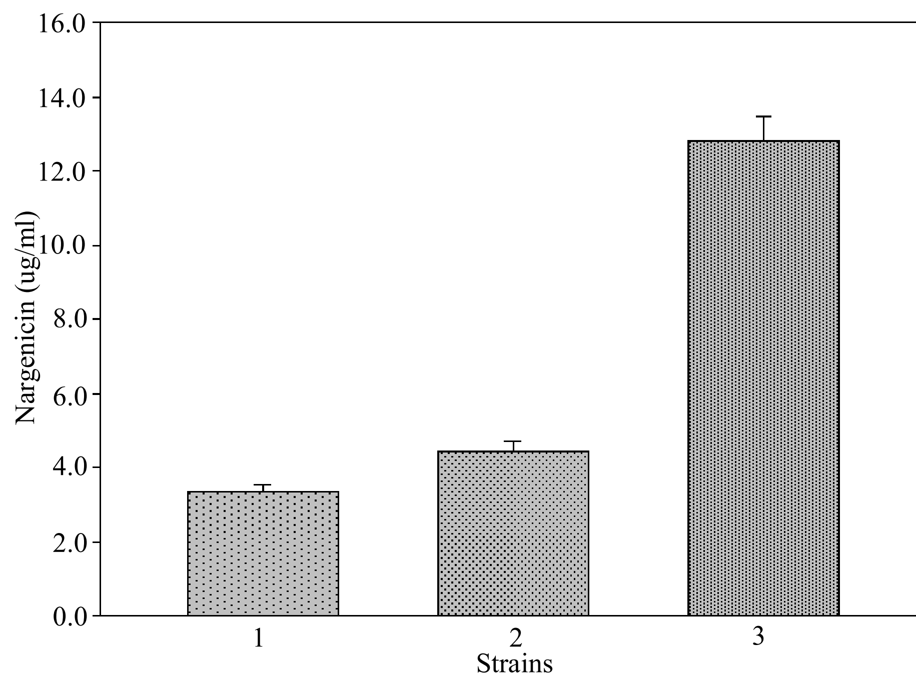


Figure 7-6. Comparison of nargenicin production by *Nocardia* sp. CS682 and its transformants. 1. Nargenicin produced by *Nocardia* wild type, 2. Nargenicin produced by *Nocardia* sp. NV18, 3. Nargenicin produced by *Nocardia* sp. ACC18.

The experiment was averaged from 4 different extractions.

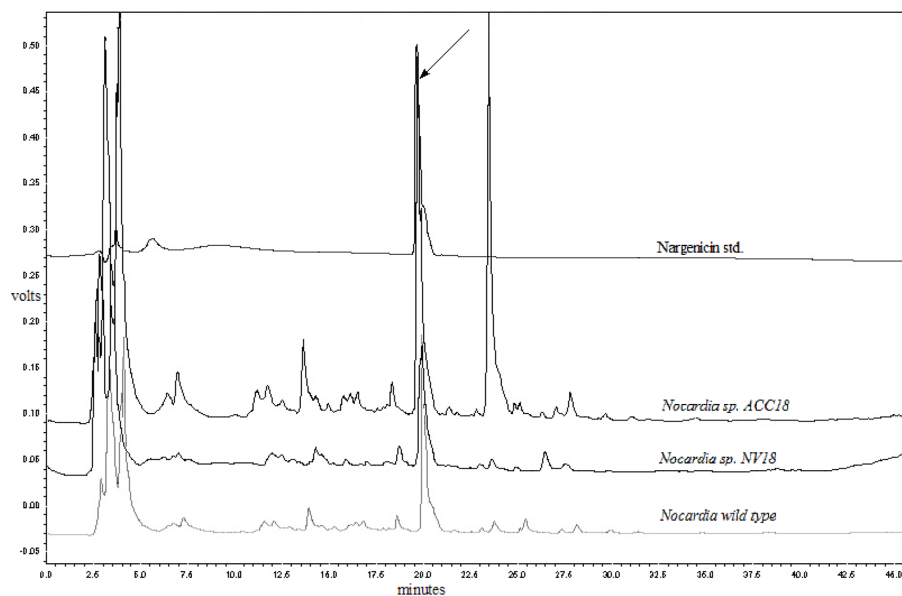


Figure 7-7. HPLC chromatogram of compounds isolated from *Nocardia* sp. CS682 and its transformants. The peak corresponding to nargenicin is indicated by an arrow head.

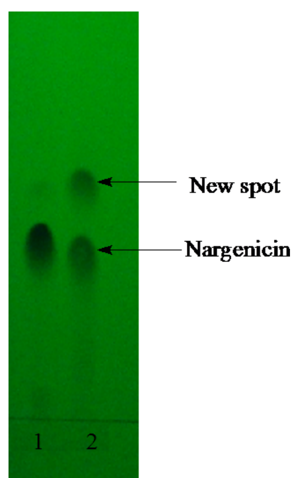


Figure 7-8. TLC assay of compounds isolated from *Nocardia* sp. ACC18.1, Standard nargenicin; 2, isolates from *Nocardia* sp. ACC18.

7.2.3 Feeding of proline

Finally we attempted to enhance the production of nargenicin by feeding proline which is proposed to be precursor of pyrrol moiety of nargenicin. Feeding experiment and analyses by HPLC and LC/MS were carried out as described in materials and methods. From this experiment we got surprising results. It was found that the production of nargenicin was observed to be decreased by about 5% as compared to that produced by *Nocardia* wild type (**Fig. 7-9**). Instead, in this experiment also, a new peak with same retention time with the peak that we observed in case of *Nocardia* sp. ACC18 was found to be increased with large extent (**Fig. 7-10**). For further confirmation, the compounds were isolated from *Nocardia* sp. ACC18 with and without feeding proline and analyzed by HPLC and TLC. These analyses revealed that in both cases the same compound has been increased. We purified this compound and analyzed by LC/MS, ^{1}H-NMR (**Fig. 7-11**) and COSY NMR (**Fig. 7-12**). ^{1}H-NMR of the compound was compared with that of nargenicin A1 as reference (**Fig. 7-13**). The molecular mass 515 and molecular formula $C_{28}H_{37}NO_8$ of the compound was similar to that of nargenicin. However, the structure of compound was slightly different from that of nargenicin (**Fig. 7-14**) and from structural analyses, we found that the new peak corresponded to the isonargenicin.

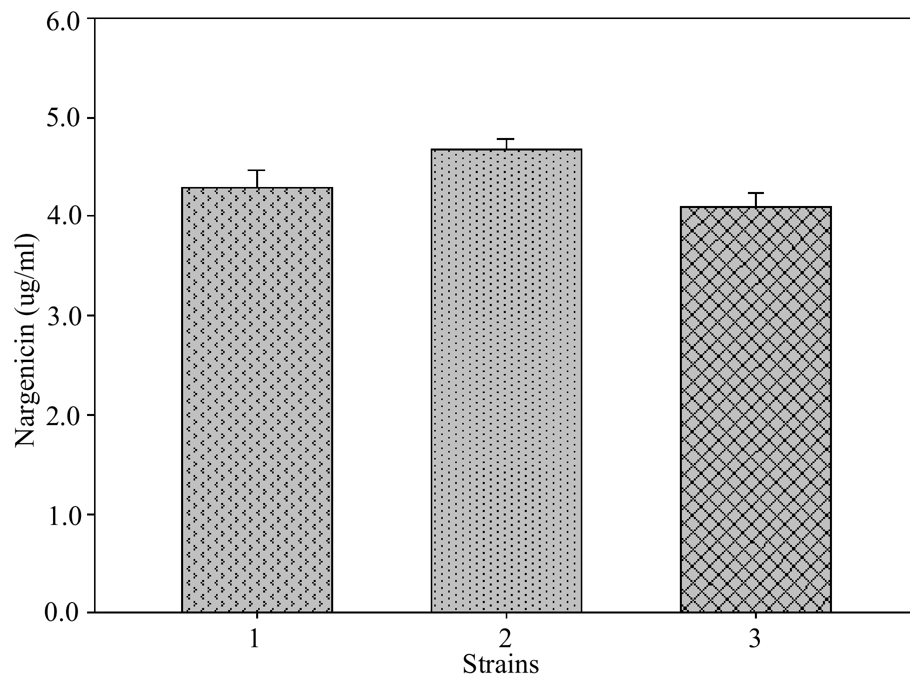


Figure 7-9. Comparison of nargenicin production by *Nocardia* sp. CS682 and its transformants after feeding proline. 1. Nargenicin produced by *Nocardia* wild type, 2. Nargenicin produced by *Nocardia* sp. NV18, 3. Nargenicin produced by *Nocardia* sp. ACC18.

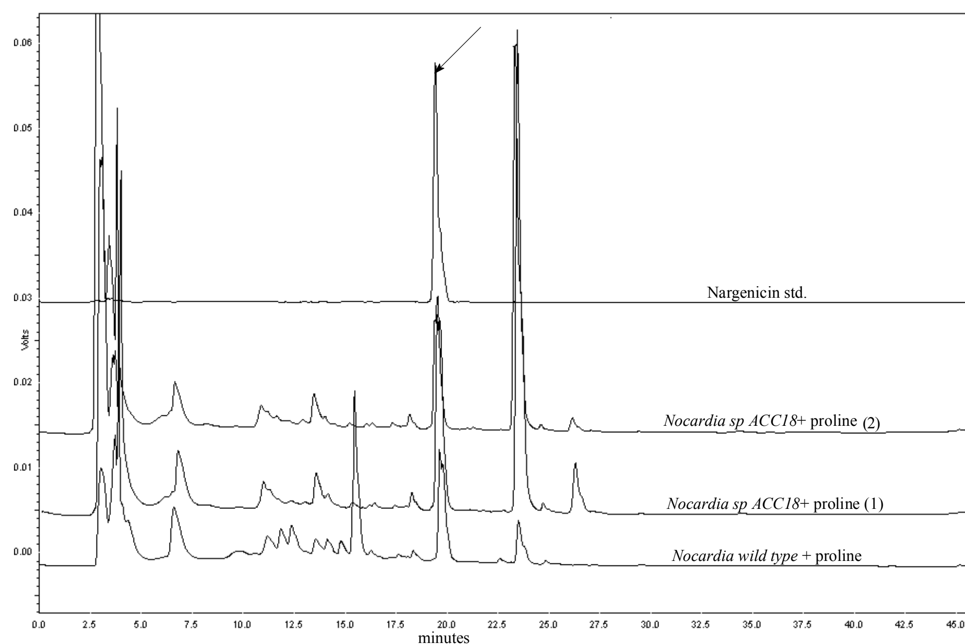


Figure 7-10. HPLC chromatogram of compounds isolated from *Nocardia* sp. CS682 and its transformants after feeding proline. The peak corresponding to nargenicin is indicated by an arrow head.

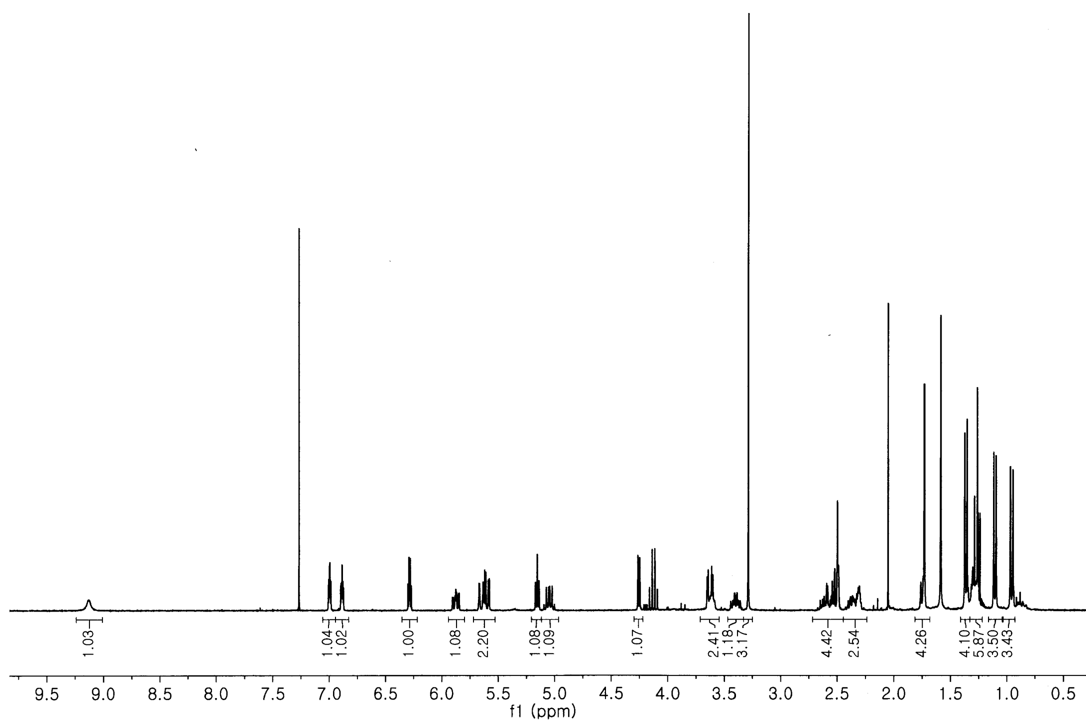


Figure 7-11. ^{1}H-NMR of isonargenicin.

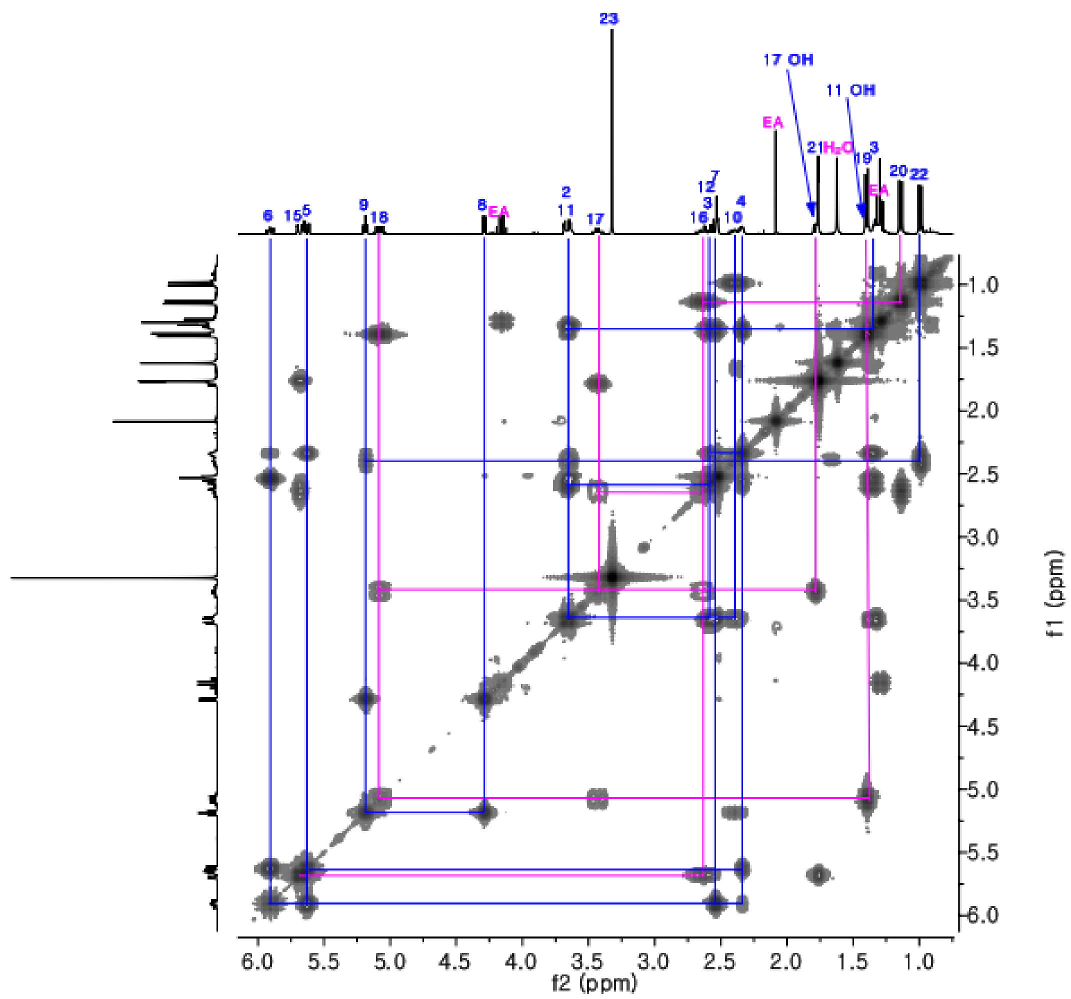


Figure 7-12. COSY-NMR of isonargenicin.

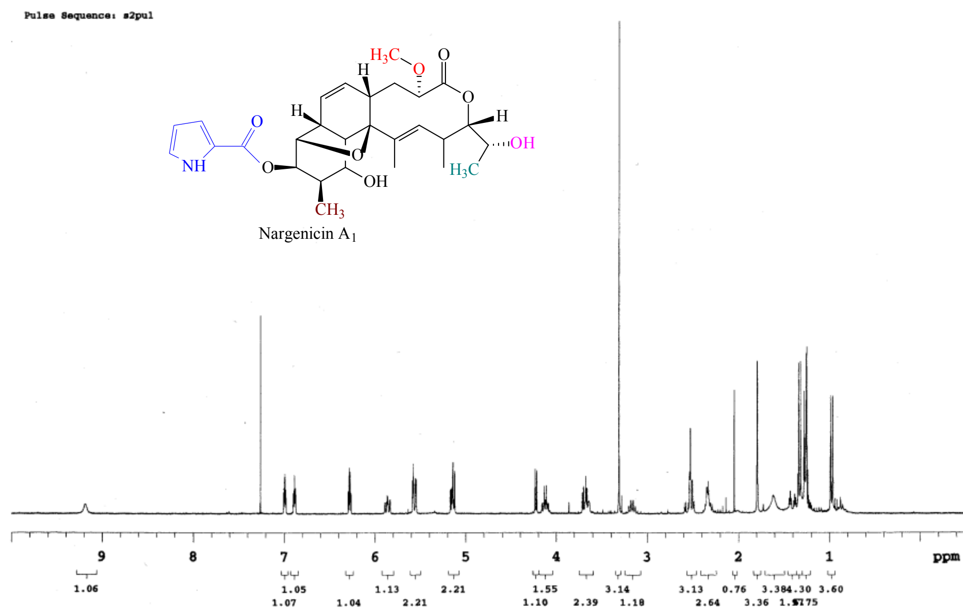


Figure 7-13. ^{1}H-NMR of nargenicin A_1.

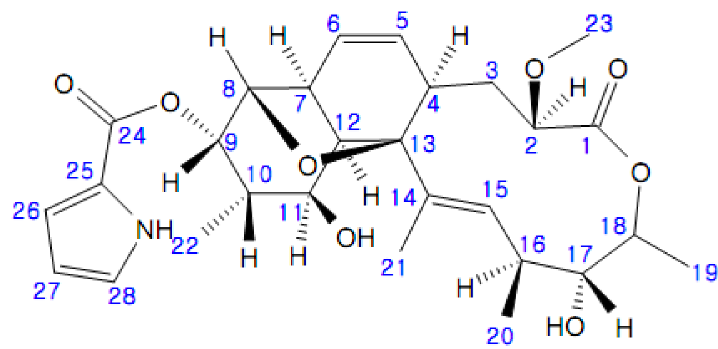


Figure 7-14. Structure of isonargenicin.

7.3 Discussion

Nargenicin A1 is therapeutically important compound since it has strong antibacterial activity against methicilin-resistant *Staphylococcus aureus* and it has the ability to induce cell differentiation, thus might be useful for the treatment of neoplastic diseases. In persuit of increased production of this therapeutically important compound we carried out heterologous expression of activator gene, *metK1-sp* and precursor genes, ACC genes. We also carried out feeding experiment of the producer strain with proline.

Several studies have shown that SAM regulates antibiotic biosynthesis in a manner independent of its role as a methyl donor, in which it acts as a direct intracellular signaling molecule for *Streptomyces* (Zhao *et al*., 2006). It also activates the transcriptional activators responsible for the induction of antibiotic synthetic genes, thereby increasing the production of antibiotics (Kim *et al*., 2003). Recent reports have suggested that SAM induces several ABC transporters in order to modulate secondary metabolism and morphological development in *S. coelicolor* (Shin *et al*., 2007). SAM also acts as a major methyl group donor for numerous transmethylation reactions. S-adenosyl-L-methionine (SAM) is synthesized from ATP and L-methionine by the action of S-adenosylmethionine synthetase (MetK). Besides, it has been proposed that C-23 methyl group of nargenicin A_1 originates from L-methionine. Thus we expressed the S-adenosylmethionine synthetase, MetK-sp from *S. peucetius* so as to increase the production of nargenicin in *Nocardia* sp. CS682. Consistent with previous studies, we found that the production of nargenicin was enhanced

by about 2.8-fold in *Nocardia* sp. metK18. The results revealed that *metK1-sp* activated the production of nargenicin in *Nocardia* sp. CS682.

Similarly, since studies have shown that nargenicin aglycon is derived from common precursors, acetate and propionate, specifically four propionate and five acetate building blocks, a gene complex for ACC was expressed in *Nocardia* sp. CS682 to enhance the carbon flux through acetyl-CoA to malonyl-CoA. The expression of ACC also enhanced the production of nargenicin in *Nocardia* sp. ACC18 by about 3.85-fold as compared to that of wild type and *Nocardia* sp. NV18. In this case, the production was higher than that in case of *Nocardia* sp. metK18.

Then after we carried out feeding experiment in *Nocardia* sp. CS682 with 3.0 g/L proline and analyzed the production of nargenicin. The proline was proposed to be the precursor of the pyrrol moiety of nargenicin and thus we presumed that expression of ACC together with proline feeding might increased the production of nargenicin to large extent. But unexpectedly, when proline was fed, the production of nargenicin was decreased by about 50% in *Nocardia* sp. ACC18 as compared to wild type without proline feeding. Then we carried out proline feeding experiment with *Nocardia* sp. CS682 in which we found that amount of nargenicin was decreased by 5% as compared to that produced by *Nocardia* sp. wild type without feeding. Instead, incase of both *Nocardia* sp. ACC18 and *Nocardia* sp. ACC18 with proline feeding, we observed a new peak in HPLC analyses which was found to be much larger as compared to that of wild type.

The structural analyses of the compound, having same molecular mass and molecular formula with that of nargenicin, revealed that it might be formed by the rearrangement of nargenicin. Thus, from this study, we concluded that the production of nargenicin is stimulated by expression of *metK1-sp* and ACC and inhibited by proline feeding whereas the production of isonargenicin is enhanced by both overexpression of ACC and feeding of proline.

Chapter VIII

Overall conclusions

Many active natural products that are used as drugs are of polyketide origin and they are synthesized by secondary metabolism processes. Although the structures of polyketides are diverse, this class of compounds is biosynthesized by condensation of carboxylic acid precursors by polyketide synthetases. Precursors for polyketide biosynthesis are generally short-chain acyl-coenzyme A (CoA) such as acetyl-CoA, propionyl-CoA, malonyl-CoA and methyl-malonyl-CoA which undergoes sequential condensation reactions catalyzed by polyketide synthases.

With the aim to enhance the production of the bioactive compounds from native producer organisms and heterologous hosts via recombinant DNA technology, we carried out metabolic engineering of *S. venezuelae* wild type, *S. venezuelae* YJ028, *S. chromofuscus* and *Nocardia* sp. CS682. Since the limitations to natural product biosynthesis might be an insufficient supply of the intracellular precursors, regulatory mechanism or biosynthetic enzymes needed to build the target natural product, our strategy was to increase the precursor pool and regulation of antibiotic production in the producer strain as well as in heterologous host.

Based on the previous studies, we began our experiment with the overexpression of positive regulator genes from *S. peucetius*, *metK1-sp* and *afsR-sp*, in *S. venezuelae*. From this study, we found that the *metK1-sp* and *afsR-sp* stimulates the regulation of pikromycin pathway regulatory gene, *pikD* as well as biosynthetic gene as a consequence of which production of pikromycin was increased by 1.6-fold and 2.6-fold respectively.

During this study, we found *afsR* homologue gene, *afsR-sv*, in *S. venezuelae* and we characterized this gene by its overexpression in native strain as well as in *S. peucetius and S. lividans* TK24. The results from this study revealed that *afsR-sv* activates antibiotic production in different *Streptomyces* strains. Pikromycin production was enhanced by approximately 4.85-fold in *S. venezuelae,* doxorubicin production was enhanced by approximately 8-fold and actinorhodin production was exhanced by approximately 1.5-fold when compared to the respective wild type strain.

Then after, we developed *S. venezuelae* YJ028, as an efficient heterologous host by expressing ACC, PCC, *metK1-sp* and *afsR-sp* separately generating four different *S. venezuelae* YJ028 mutant hosts so as to increase the precursor pool to be directed towards enhanced production of various polyketides. The efficacy of the developed hosts was studied by overexpressing type III polyketide synthase, 1,3,6,8-tetrahydroxynaphthalene synthase, in these hosts. We observed that flaviolin production was doubled by expression of ACC and increased 4-fold by combined expression of ACC, *metK1-sp* and *afsR-sp*.

But when we overexpressed these precursor and regulatory genes in *S. chromofuscus* in order to enhance herboxidiene production, unexpectedly and irrespective of the genes expressed, all *S. chromofuscus* mutant strains showed change in morphology and produced blue and red pigmented compounds, with the abolishment of herboxidiene production. From complete analyses of blue and one of the red compounds, we found that all these strains produced blue pigment antibiotic, actinorhodin and red colored immunosuppressive drug, undecylprodigiosin.

However, when only *metK1-sp* was expressed in *Nocardia*, nargenicin was found to be enhanced by about 2.8 times and when ACC was expressed, the production of nargenicin was increased by about 3.85 times. But when proline was fed, the nargenicin production was decreased. Besides, when ACC was overexpressed, we observed that a new peak corresponding to another compound was found to be increased much more than nargenicin as revealed by HPLC analyses. This compound was purified, analyzed by LC/MS and NMR and found that it was isonargenicin. Thus, the production of nargenicin is stimulated by expression of *metK1-sp* and ACC and inhibited by proline.

In conclusion, through metabolic engineering of native as well as heterologous host the production of bioactive compounds can be enhanced. Furthermore, we can apply this approach to stimulate the biosynthesis of many therapeutically important compounds which remain inactive in native state.

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(국문요약)

ACTINOMYCETES 의 대사공학 연구**:** ***Streptomyces*** 와 ***Nocardia***

균주로부터 **POLYKETIDE** 의 생산성 증가를 위한 연구

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대사공학 연구들은 임상적으로 중요한 polyketide 생산을 향상시키기 위해 동종 균주의 유전자 조작 및 이종 숙주 개발 등 다양한 연구가 수행되고 있다. 생합성 과정에서 대사 흐름 개선, 전구체 공급 향상 및 부산물 합성 감소 등 유사한 분자 생물학적 기술들을 원 숙주와 이종 숙주에 사용하였다. 본 연구에서는

생물활성이 있는 polyketide 생산을 향상시키기 위해 동종 생산 균주의 유전자 조작과 이종숙주를 사용하여 성공적으로 수행 되었다.

본 연구는 *Streptomyces* 와 *Nocardia* 균주로 부터 생물활성이 있는 이차 대사산물 생산을 동종과 이종숙주에서 향상시키기 위해 두 균주의 대사 공학 연구에 초점을 맞추었다. Pikromycin 생산균주에서 생산성을 향상시키기 위해 *S. peucetius* ATCC 27952 로부터 확보된 MetK 단백질과 포괄적인 조절 단백질에 해당하는 유전자인 *metK1-sp* 와 *afsR*-sp 두개의 양성 조절 유전자를 *S. venezuelae* ATCC 15439 이종숙주에서 발현 하였다. 그 결과 pikromycin 생산은 *metK1-sp* 와 *afsR*-sp 발현에 의해 각각 1.6 배와 2.6 배 증가하였다. *S. venezuelae* 에서 *metK1-sp* 와 *afsR-sp* 의 발현은 RT-PCR 에 의해 이미 증명된 경로-특이적 조절 유전자인 *pikD* 와 생합성 과정 유전자인 ketosynthase 유전자 발현에 영향을 준다. *pikD* 와 ketosynthase 유전자의 높은 전사율은 pikromycin 생산증가와 일치한다. (Chapter III)

S. venezuelae ATCC 15439 게놈 분석에서 우리는 포괄적인 조절 단백에 해당하는 3.1-kb open reading frame (ORF)를 얻었으며, 이 *orf* 를 *afsR-sv* 로 명명하였다. *afsR-sv* (1,056 aa)는 이미 잘 알려져 있는 포괄적인 조절 단백질 AfsR 과 매우 높은 상동성을 가지고 있다. 상동성-근거로 실험을 한 결과 발현된 *afsR-sv* 도 *Streptomyces* 항생제 조절 단백질(SARP)과 관련된 전사적 활성을 보여준다. 이 SARP 는 박테리아 전사 활성 도메인(BTAD), NB-ARC 도메인 그리고 C-말단 tetratricopeptide 반복 도메인을 포함하는 N-말단 SARP 도메인으로 이루어져 있다. 우리는 *S. venezuelae* 균주에서 임상적으로 중요한 polyketide 생산을 향상하기 위해 *S. venezuelae* 균주에서 클론된 포괄적인 조절 유전자 (*afsR-sv*)를 사용하였다. *afsR-sv* 가 발현된 균주는 (*S. venezuelae*/pASV152) 야생형 *S. venezuelae* 와 비교하였을 때 약 4.85 배 증가된 pikromycin 생산을 보였다. 역전사 PCR (RT-PCR)에 의한 pikromycin 생합성 유전자 발현 분석은 *afsR-sv* 가 *S. venezuelae* 내에서 발현 되었을 때, pikromycin 생산에 관련된 유전자의 전사가 보다 활성되어 있음을 확인하였다. 유사한 방법으로 다른 *Streptomyces* 균주

내에서 *afsR-sv* 의 발현은 각 해당하는 항생제 생산이 증가하는 결과로 나타났다. 이는 *afsR-sv* 가 항생제 생합성의 양성 조절자임을 보여준다. *S. peucetius*/pASV25 에 의해 생산된 doxorubicin 의 양은 야생 균주에서 생산된 것 보다 약 8.04 배 더 높았고, *S. lividans* TK24/pASV25 에 의해 생산된 actinorhodin 의 양은 야생 균주 보다 약 1.5 배 높았다. (Chapter IV)

이차 대사산물의 생산을 향상시키기 위해 동종생산 균주에서 성공적인 결과를 바탕으로, 효과적인 이차 대사산물의 생산을 위한 이종 숙주 개발을 하였다. 이종 숙주 생산균주 개발은 생물활성 이차 대사산물을 생산하는 균주들 중에 많은 균주들이 배양이 어렵고, 성장이 느리고, 일부 미생물들은 유전적으로 조작하기가 어렵기 때문이다. 어떤 경우에는 미생물의 생합성 경로 네트워크의 복잡성 때문에 다중 조절인자의 조작에 의한 예상했던 결과물을 얻기가 어렵다.

대사 공학 방법을 사용하여 우리는 다양한 polyketide 생산을 향상하는 방향으로 유도하기 위해 전구체의 양이 증가 되도록 효율적인 *S. venezuelae* YJ028 균주를 개발하였다. 본 연구에서는 acetyl-CoA carboxylase, propionyl-CoA

carboxylase, *metK1-sp* 와 *afsR-sp* 유전자가 발현할 수 있도록 *S. venezuelae* YJ028 에 전이하여 네개의 균주를 개발하였다. 새로이 개발된 이종숙주에서 polyketide 생산성을 확인하기 위해, polyketide synthase 중에 가장 단순한 polyketide synthase type Ⅲ 계열인 1,3,6,8-tetrahydroxynaphthalene synthase (flaviolin 합성효소) 유전자를 발현하였다. Flaviolin 생산은 acetyl-CoA carboxylase (ACC)의 발현의 경우 두 배 증가하였다. 그리고 ACC, *metK1-sp* 와 *afsR-sp* 의 조합하여 발현한 경우 4 배 증가하였다. 따라서, 새로이 개발된 *S. venezuelae* YJ028 숙주는 원균주 보다 효율적으로 polyketide 를 생산한다. (Chapter V)

환경친화적인 제초제인 herboxidiene 생산성을 증가시킬 목적과 herboxidiene의 새로운 유도체를 합성하기 위해, 메틸 그룹 공여체 *metK1-sp* 및 포괄적인 조절 유전자 *afsR-sp*와 함께 acetyl-CoA carboxylase (ACC) 와 propionyl-CoA carboxylase (PCC) 유전자의 발현할 수 있도록 herboxidiene 생산균주인 *S. chromofuscus* ATCC 49982 대사 공학을 수행하였다. 이들 유전자가 발현한 후, herboxidiene 합성은 안되고, *S. chromofuscus* 돌연변이 균주들의

표현형(morphology)이 변화된 것을 보였고, 예상치 않은 푸른색 및 붉은색을 가진 화합물이 생산된다. 푸른색과 붉은색 화합물을 분석한 결과, 푸른색을 갖는 화합물은 actinorhodin 항생제로 확인 되었으며, 붉은색을 갖는 물질은 면역 억제성 약제인 undecylprodigiosin 으로 확인하였다. (Chapter VI)

Nocardia sp. CS682에서 nargenicin A1 생산을 증가시키기 위해, 우리는 *metK1-sp* 와 acetyl-CoA carboxylase 각각 발현과 proline 공급을 함께 수행 하였다. *metK-sp*가 발현되었을 때, nargenicin 은 *Nocardia sp.* CS682 와 *Nocardia sp* NV18 와 비교하여 *Nocardia sp* metK18 에서 약 2.8배 증가 된 것이 확인 되었다. Acetyl-CoA carboxylase 가 발현 되었을 때, *Nocardia sp* ACC18에서 nargenicin 의 생산이 *Nocardia sp.* CS682 와 *Nocardia sp* NV18 에 의해 생산 된 것 보다 약 3.85배 증가 된 것을 확인하였다. 그러나 뜻밖에도 3.0g/L proline이 배지에 부가되었을 때 nargenicin 의 생산은 proline 이 공급되지 않은 균주와 비교하여 *Nocardia sp* ACC18에서 약 50% 감소 하였다. Proline을 공급된 것과 공급되지 않은 *Nocardia sp* ACC18로부터 화합물을 분리 및 분석하였을 때, 또 다른 화합물로

상응하는 피크가 HPLC 분석결과 nargenicin 보다 훨씬 더 증가 된다. LC/MS, ^{1}H-NMR, ^{13}C-NMR 와 COSY에 의한 화합물의 정제 및 분석결과 생산이 증가된 이 화합물은 isonargenicin 으로 확인되었다. 따라서, 본 연구 결과는 nargenicin의 생산이 *metK1-sp* 와 acetyl-CoA carboxylase의 발현에 의해 증가되지만, proline 공급은 생산량 억제가 일어남을 결론지었다. 대신에, isonargenicin의 생산이 acetyl-CoA carboxylase가 발현된 경우와 proline 이 공급되어졌을 때 nargenicin 과 비교해 보면 훨씬 더 증가되는 것으로 확인하였다. (Chapter VII)

핵심어: 대사 공학, *metK1-sp, afsR-sp,* acetyl-CoA carboxylase, propionyl-CoA carboxylase, *S. venezuelae* YJ028, pikromycin, *S. chromofuscus* ATCC 49982, herboxidiene, actinorhodin, undecylprodigiosin, *Nocardia sp.* CS682, nargenicin

Appendix

LIST OF PUBLICATIONS:

1. **Sushila Maharjan**, Je Won Park, Yeo Joon Yoon, Hei Chan Lee, Jae Kyung Sohng. Metabolic engineering of *Streptomyces venezuelae* for malonyl-CoA biosynthesis to enhance heterologous production of polyketides. *Biotechnol Lett*. (2010), 32(2): 277–282.

2. **Sushila Maharjan**, Tae-Jin Oh, Hei Chan Lee, and Jae Kyung Sohng. Identification and functional characterization of an *afsR* homolog regulatory gene from *Streptomyces venezuelae* ATCC 15439. *J. Microbiol. Biotechnol.* (2009), 19(2): 121–127.

3. **Sushila Maharjan**, Tae-Jin Oh, Hei Chan Lee, and Jae Kyung Sohng. Heterologous expression of *metK1-sp* and *afsR-sp* in *Streptomyces venezuelae* for the production of pikromycin. *Biotechnol Lett* (2008), 30(9), 1621–1626.

POSTER PRESENTATIONS:

1. **Sushila Maharjan**, Jae Kyung Sohng and Hei Chan Lee. Identification & Functional Analysis of *afsR* Homologue Regulatory Gene from *Streptomyces venezuelae* 15439, 2008 KSPE SPRING MEETING AND CGMP SYMPOSIUM. May 18–19, 2007, SUNMOON UNIVERSITY, ASAN, Republic of Korea. Poster Number- PP23 (page 115).
2. **Sushila Maharjan**, Jae Kyung Sohng and Hei Chan Lee. Identification & Functional Analysis of *afsR* Homologue Regulatory Gene from *Streptomyces venezuelae* 15439, 2008. 36th KSIEC Meeting. November 2–3, 2007, Hankyong National University, Anseong, Republic of Korea. Poster Number- 1P-3 (page 81).
3. **Sushila Maharjan**, Jae Kyung Sohng and Hei Chan Lee**.** Development of *Streptomyces venezuelae* YJ028 as a specific host bacteria for polyketide production, 2008 KSPE SPRING MEETING AND CGMP SYMPOSIUM. May 16-17, 2008, SUNMOON UNIVERSITY, ASAN, Republic of Korea. Poster Number- PP22 (page 48).
4. **Sushila Maharjan**, Hei Chan Lee, Kwangkyoung Liou, and Jae Kyung Sohng. Metabolic engineering of *Streptomyces venezuelae* YJ028 for malonyl CoA

biosynthesis to enhance heterologous production of polyketides. The 5th Japan-Korea Chemical Biology Symposium, The Westin Chosun Hotel, Busan, Korea (ROK), January 26–28, 2010. Poster No. P-14, page 38.

5. **Sushila Maharjan**, Hei Chan Lee, Kwangkyoung Liou, and Jae Kyung Sohng. Metabolic engineering of *Streptomyces venezuelae* YJ028 for malonyl CoA biosynthesis to enhance heterologous production of polyketides. Poster presented at MSK's (The Microbiology Society of Korea) 50th Anniversary International Symposium on Microbiology 2009. May 28–30, 2009, Poster No. P-G021, Jeju Island, Korea.