공 학 박 사 학 위 논 문

Engineering of Acyl-CoA Metabolism for Production of Branched Chain Fatty Acids and Pikromycin in *Streptomyces*

방선균에서 Acyl-CoA 대사 조절을 통한 가지사슬형 지방산 및 Pikromycin 생산연구

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공과대학 화학생물공학부

이 정 상

Engineering of Acyl-CoA Metabolism for Production of Branched Chain Fatty Acids and Pikromycin in *Streptomyces*

A Thesis
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by

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Abstract

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In this thesis, branched chain fatty acids (BCFA) and polyketide antibiotics, pikromycin (PKM), were overproduced in *Streptomyces* species. Primarily, branched chain amino acids (BCAA) catabolism were examined and engineered for synthesis of BCFA in *Streptomyces coelicolor*, and PKM in *Streptomyces venezuelae*.

Increases in productions of BCFAs were achieved by overexpressing branched chain α-keto acid dehydrogenase (BCDH) and 3-ketoacyl acyl carrier protein synthase III (KASIII) in polyketide synthase gene clusters deleted *S. coelicolor* strain. Deletion of *fadD* of β-oxidation was also performed to block degradation of BCFAs.

For enhancing productions of PKM from *S. venezuelae*, three key enzymes in BCAA catabolism, KASIII, acyl-CoA dehydrogenase (ACAD), and BCDH were manipulated. First, using promoter and 5'UTR sets developed in *S. coelicolor*, KASIII was repressed to lower fatty acid (FA) synthesis, but to increase polyketide synthesis on the other hand. Unfortunately, growth defects from KASIII repression was too severe that the PKM production decreased. As a result, BCDH and ACAD were overexpressed to increase the fluxes towards methylmalonyl-CoA productions. It was compared to PKM productions from central carbon metabolism with Methylmalonyl-CoA mutase (MCM) overexpression. It appeared that methylmalonyl-CoA productions from central carbon metabolism was more effective than that from BCAA catabolism. When BCDH and MCM were overexpressed together, precursor supplementation was no longer a bottleneck, but activities of pikC P450 monooxygenase limited synthesis of pikromycin.

In short, this study explored less-assessed area of BCAA metabolism by engineering, to generate precursors of polyketide antibiotics and fatty acids. It would provide general insights to design methodologies for productions of various metabolites composed of acyl-CoAs in various *Streptomyces* sp..

Keywords: *Streptomyces*, promoter engineering, pikromycin, branched chain amino acids, branched chain fatty acids, polyketide synthase genes, fatty acid synthase genes, β-oxidation.

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Introduction

1.1 Polyketide and fatty acids synthesis in *Streptomyces* species

1.1.1 Polyketide synthase genes

Soil-dwelling microorganism *Streptomyces* species are responsible for productions of numerous secondary metabolites (Yi et al., 2017). About 7600 different antibiotics and antitumor drugs are known to be produced by this organism (Berdy, 2005). Among them, polyketides are one of the most well-known antibiotics. Each of *Streptomyces* sp. are evolved into producing different types of polyketide antibiotics, but basic structures of them are the same (Plater and Strohl, 1994). Using acyl-CoA, both as a starter and an elongation unit, polyketide synthase (PKS) genes produce polyketides in various structures.

There are three types of PKS. Type I PKS genes are clustered into one modular enzymes. During polyketide chain elongation, productions of polyketides can be either completed with one cycle, or repeated with the same PKS genes (Cheng et al., 2009). Type II PKS genes are similar to that of type I, but type II PKS genes are subdivided into smaller mono-functional clusters (Hertweck et al., 2007). Type III PKS genes are somewhat different from type I and II. Type III is in a form of a homodimer, where one domain performs functions of multiple domains (Shimizu et al., 2017). PKS modules are composed of generally seven different domains (Figure 1.1).

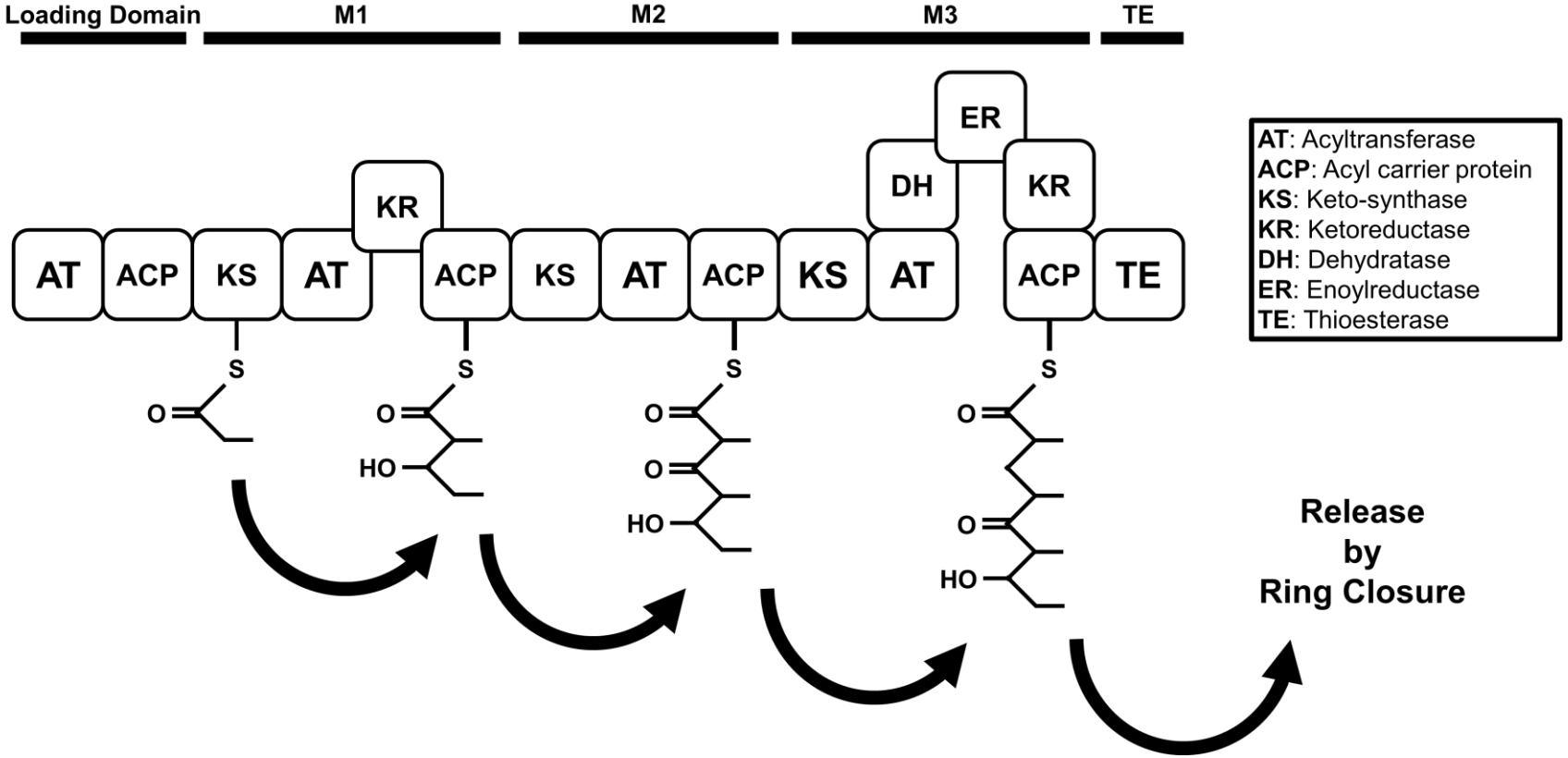
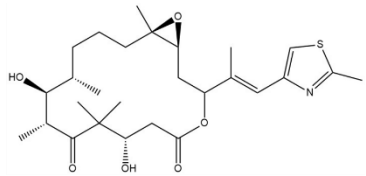


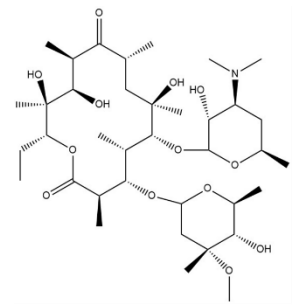
Figure 1.1. Basic scheme of type I and II polyketide synthase genes.

Each modules are composed of acyltransferase (AT), acyl carrier protein (ACP), keto-synthase (KS), ketoreductase (KR), dehydratase (DH), enoylreductase (ER), and thioesterase (TE). AT transfers acyl group from acyl-CoAs to ACP. KS domain performs condensation reaction of acyl-ACP with other acyl-CoAs (i.e. malonyl-CoA and methylmalonyl-CoA), performing chain elongations of polyketides (Yu et al., 2012). The other modules are related to modifications of the acyl-chains and termination of chain elongations. KR reduces keto groups into hydroxyl groups, and DH removes the hydroxyl groups in forms of water. ER reduces unsaturatedness, converting a carbon to carbon double bond into a single bond. TE is responsible for the termination of chain elongations, by releasing acyl-chains from ACP by ring-closing reaction (Gokhale et al., 1999). It makes TE determine chain lengths and ring sizes of polyketides. And then, polyketides go through post PKS tailoring steps.

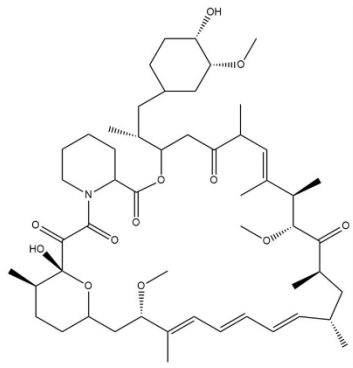
Post tailoring enzymes are included in a PKS gene cluster, but are expressed, regulated, and perform independently from that of PKS genes (Rex et al., 2002). Modifications include glycosylation and dimerization, resulting in various polyketide metabolites (Figure 1.2).



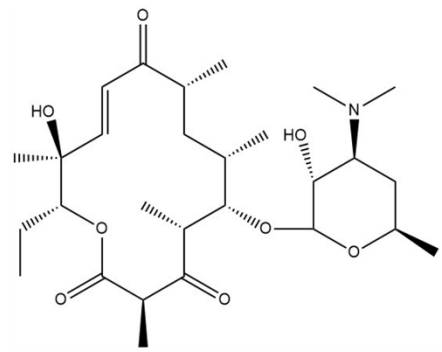
Epothilone



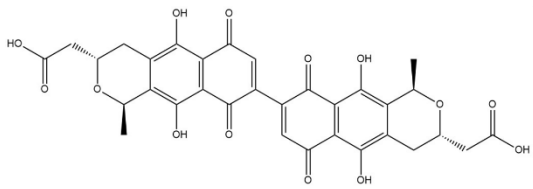
Erythromycin



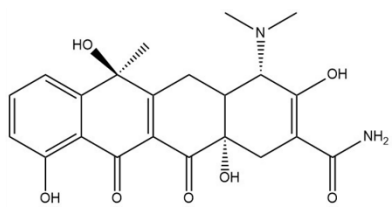
Rapamycin



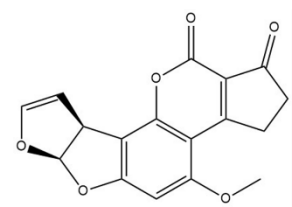
Pikromycin



Actinorhodin



Tetracycline



Aflatoxin B1

Figure 1.2. Various polyketide antibiotics and anticancer drugs.

1.1.2 Fatty acid synthase genes

Fatty acid biosynthesis is very similar to that of polyketides. There are two types of fatty acid synthase (FAS) genes, type I and type II. Just as in PKS, type I FAS is composed of a single large enzyme with multiple functionalities. Type II is also composed of several enzymes with individual functions (Janβen and Steinbuchel, 2014).

For the synthesis of fatty acids, the same reduction cycles of acyl-CoA on ACP of FAS occur as they did with PKS genes. Fatty acid biosynthesis starts with transfer of acyl group from malonyl-CoA to ACP, resulting in malonyl-ACP, by ACP transacylase. And then, β-ketoacyl-ACP synthase adds acetyl-CoA, creating acetoacetyl-ACP (Jackowski and Rock, 1987). Chain elongation steps occur afterwards, using malonyl-CoA as an elongation unit. KR, DH, and TE of FAS genes perform the same reactions as those from PKS genes, resulting in long and linear hydrocarbon chain of carboxylic acids in various lengths (Lennen and Pfleger, 2012) (Figure 1.3a). TE of FAS gene is also responsible for lengths of the hydrocarbon chains (Cantu et al, 2010). Such similarities allowed productions of various fatty acids from PKS genes, and vice versa in recent studies (Hagen et al., 2016; Gajewski et al., 2017).

Differences between PKS and FAS genes are that keto groups of acyl-CoA are removed in forms of water for every step of condensation reactions, and TE releases linear hydrocarbon chain in fatty acids biosynthesis. There are little variations due to unsaturatedness and linearities in the hydrocarbon chains (Cantu et al, 2010).

Another differences is a presence of a fatty acid degradation pathway, β -oxidation (Figure 1.3b). Fatty acids are degradated into individual acetyl-CoAs per cycle in β-oxidation. It can be stated as a reverse of FAS. The β -oxidation cycle is consisted of, i) dehydrogenation of C2 and C3 of fatty acids, ii) hydration and dehydrogenation generating a keto group on C3 position, and iii) thiolation with CoA resulting in C_{n-1} fatty acid and an acetyl-CoA (Iram and Cronan, 2006). For productions of biofuels with fatty acids, this pathway is often deleted in production hosts to stop degradations of the end products (Lennen and Pfleger, 2012).

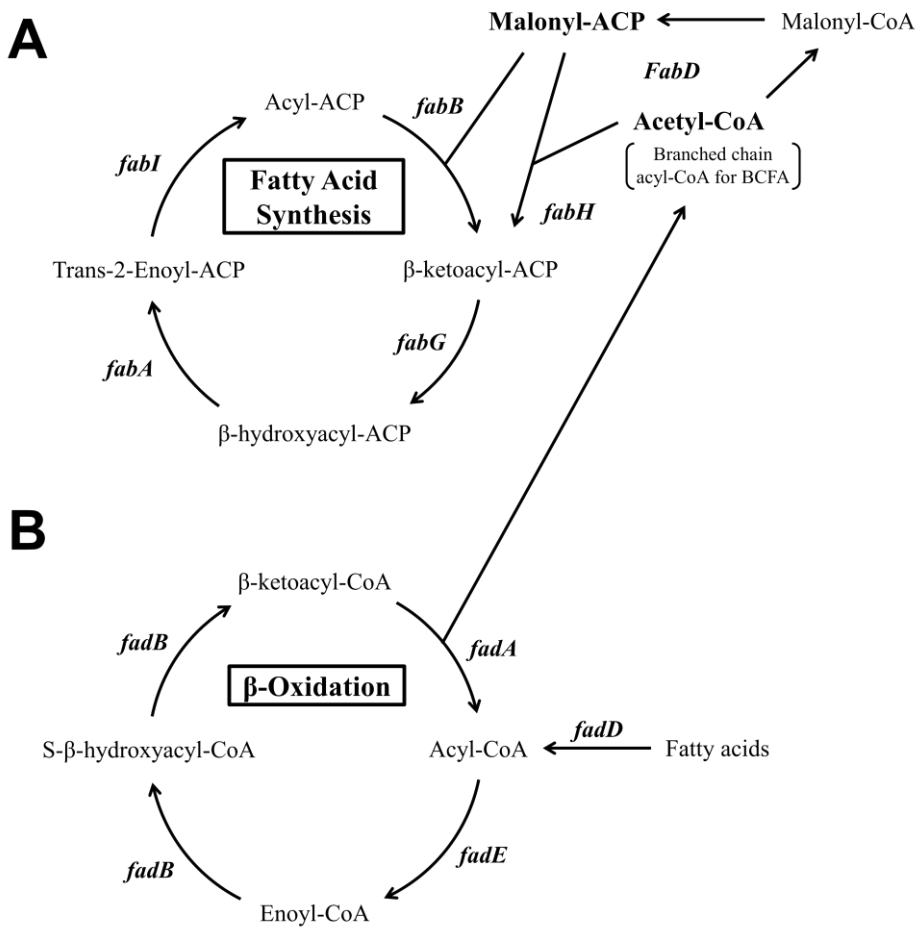


Figure 1.3. (a) Fatty acids synthesis pathway, and (b) its degradation pathway, β-oxidation.

1.2 Synthesis of precursors and co-factors

1.2.1 Acyl-CoA productions from carbon and nitrogen metabolism

Acyl-CoA precursors for fatty acids and polyketides come from both carbon and nitrogen metabolism (Figure 1.4). From the central carbon metabolism, acetyl-CoA from pyruvate, and succinyl-CoA from α-ketoglutarate are mainly produced in large quantities (Parimi et al., 2017). Glucose is catabolized into pyruvate through glycolysis, and pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA then can be further metabolized through tricarboxylic acid (TCA) cycle, or converted to malonyl-CoA by acetyl-CoA carboxylase (ACC), which is used for both fatty acids and polyketides synthesis.

More variable acyl-CoA compounds are produced by amino acids metabolism. For example, acetyl-CoA, propionyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA could be produced from branched chain amino acids, which are leucine, isoleucine, and valine (Erb et al., 2009). Glutamate is converted into succinyl-CoA via TCA cycle.

Metabolic pathways for acyl-CoA metabolism is in a form of network. One is connected to the other in many different ways that it is hard to determine which nutrient results in which acyl-CoA compounds. But it is possible to have a glimpse of the sources of target acyl-CoA compounds with media optimizations and nutrient feeding.

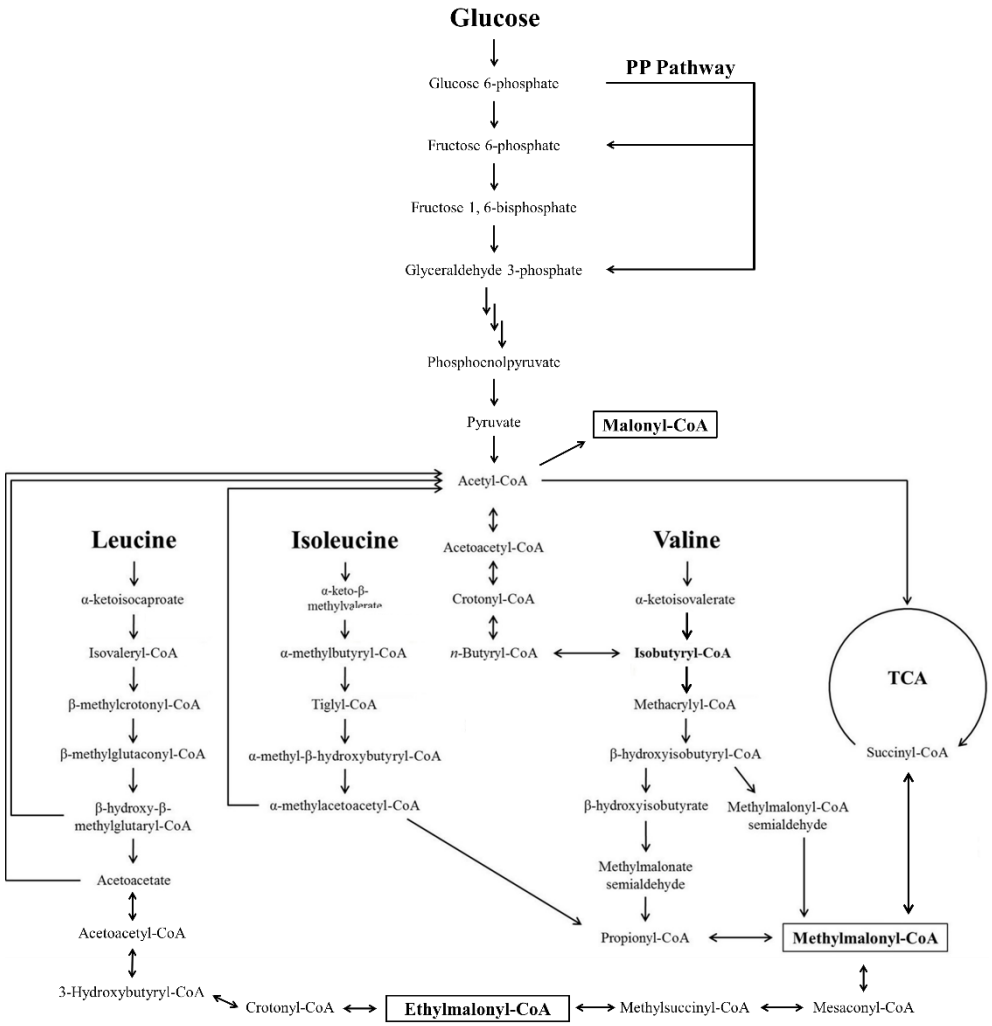


Figure 1.4. Pathway of glucose and branched chain amino acid metabolism.

1.2.2 Cofactor NADPH productions, consumptions, and regenerations

Polyketides and fatty acids biosynthesis requires NADPH as a cofactor during elongation steps. Reductases in PKS and FAS genes, KR and ER, utilize NADPH to reduce keto groups into hydroxides (Korman et al., 2004; Caffrey, 2012). As a result, NADPH pools are often increased along with acyl-CoA precursors for overproductions of polyketides and fatty acids (Kim et al., 2016). Such NADPH is produced from NAD/NADH, which are produced from tryptophan, nicotinic acid, and nicotinamide (Mattevi, 2006).

NADPH can be produced from both NAD^+ and NADH. A conversion of NAD^+ into $NADP^+$ is performed by NAD kinase. NAD kinase phosphorylates at 2' of NAD^+ ribose ring using ATP as a donor (Kawai et al., 2001). And then, resulting $NADP^+$ is reduced to NADPH. It applies the same for NADH, where NADH kinase phosphorylates NADH into NADPH (Outten and Culotta, 2003).

There are three major sources for NADPH productions, 10-formyl-THF pathway, pentose phosphate pathway (PPP), and malic enzyme (Figure 1.5). Roughly 50% of NADPH are reported to be produced during purine biosynthesis from serine and THF (Fan et al., 2014). And the rest are from malic enzyme of TCA cycle, and PPP. Through these pathways, $NADP^+$ are reduced and recycled back to NADPH for uses in PKS and FAS genes.

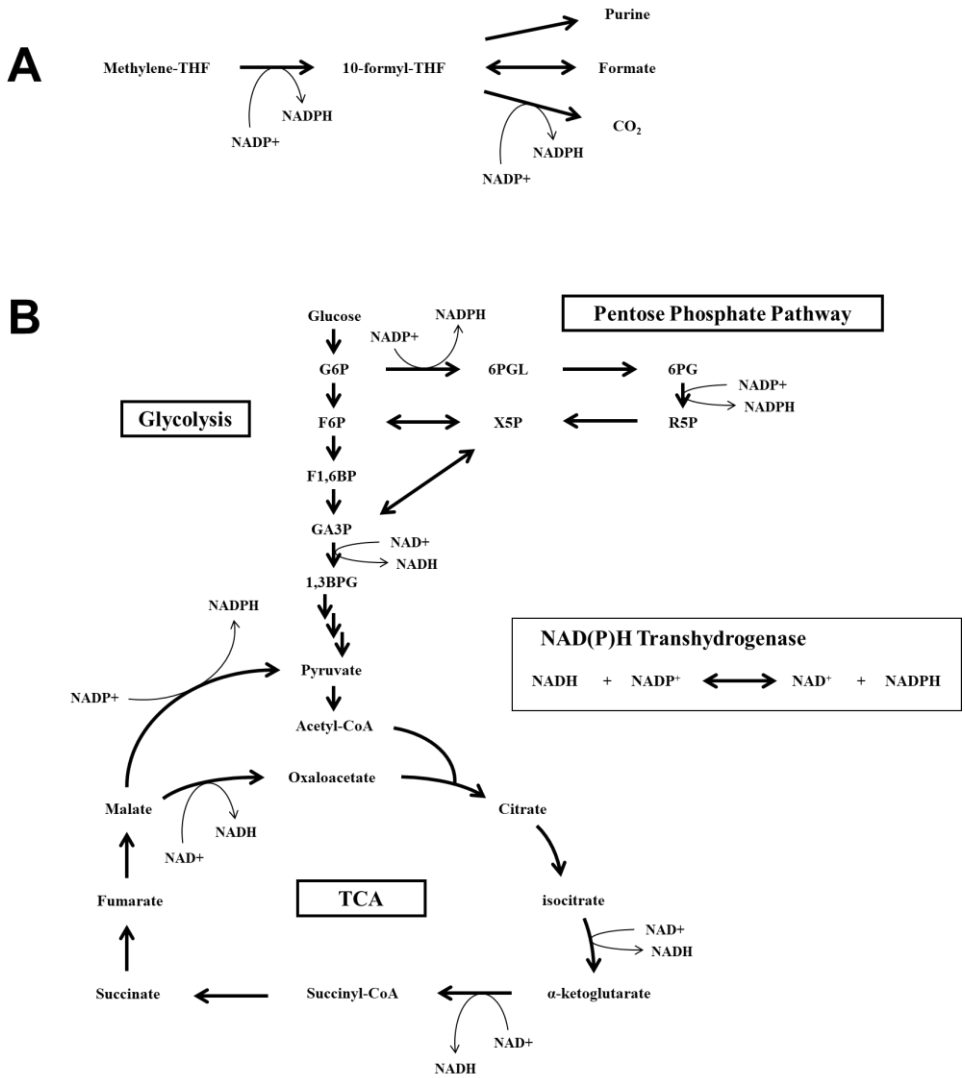


Figure 1.5. NADH and NADPH cofactor regenerations from (a) 10-formyl-THF pathway, and (b) glycolysis, pentose phosphate pathway, and TCA cycle.

1.2.3 Strategies to increase acyl-CoA pools

Increases in polyketide antibiotics are often achieved by increased supply of acyl-CoA precursors. There are three major building blocks of polyketides, which are malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA (Chan et al., 2009; Olano et al., 2008). Carbon and nitrogen metabolisms, i.e. glycolysis and amino acids metabolism, are major metabolic pathways for supplementation of such CoA precursors. For that reason, key enzymes participating in the pathways are overexpressed or engineered to increase conversion of C and N sources into acyl-CoAs.

A major strategy to produce malonyl-CoA is to increase carbon fluxes towards glycolysis. Simple overexpression of ACC resulted increases in malonyl-CoA pools because malonyl-CoA is produced from carboxylation of acetyl-CoA by ACC (Davis et al., 2000). In addition to ACC overexpression, reducing carbon fluxes into pentose phosphate pathway (PPP) and TCA cycle resulted further increases in malonyl-CoA productions. Deletions of glucose-6-phosphate 1-dehydrogenase isomers (Ryu et al., 2006), or overexpressions of pyruvate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase (Bhan et al., 2013) reduced carbon fluxes into PPP. And in return, it redirected the carbon fluxes towards glycolysis for acetyl-CoA. Down regulations of genes participating in TCA cycle, major acetyl-CoA consuming pathway, accumulated pyruvate and actyl-CoA, which also yielded increased malonyl-CoA pools (Bhan et al., 2013) (Figure 1.6).

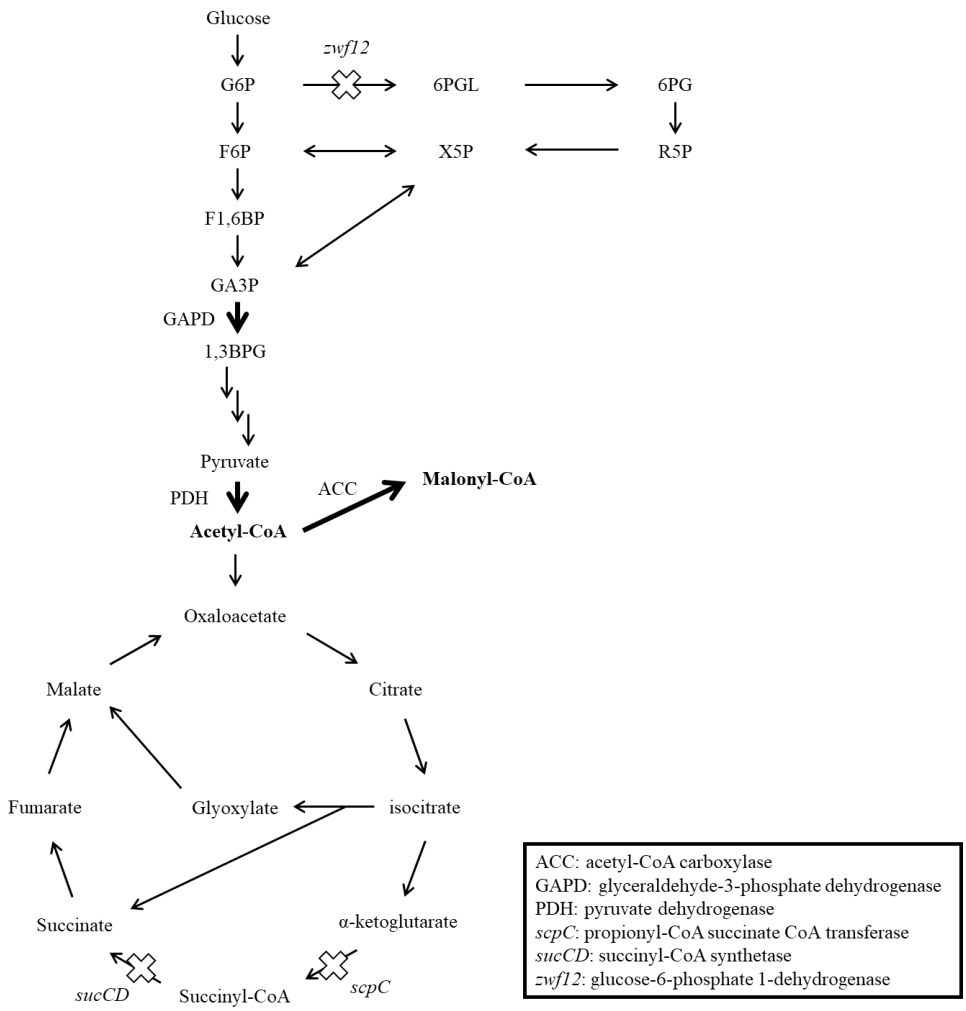


Figure 1.6. Strategies to increase productions of acetyl-CoA and malonyl-CoA for productions of polyketide secondary metabolites. Overexpression targets are marked by bold arrow, and deletion targets are marked by X.

Another pathway producing malonyl-CoA is a metabolism of branched chain amino acids (BCAA). In *Streptomyces coelicolor*, BCAA is responsible for 50% of malonyl-CoA for polyketide antibiotics, actinorhodin (ACT) (Stirrett *et al*., 2009). Overexpressions of branched chain α-keto acid dehydrogenase (BCDH) resulted about 52 fold increases in ACT productions (Kim et al., 2014). Productions of methylmalonyl-CoA and ethylmalonyl-CoA are overproduced in a similar manner.

Methylmalonyl-CoA is directly produced from either propionyl-CoA by propionyl-CoA carboxylase (PCC), or succinyl-CoA by methylmalonyl-CoA mustase (MCM). Ethylmalonyl-CoA is produced by crotonyl-CoA reductase (CCR) from crotonyl-CoA, or multiple enzymatic steps from methylmalonyl-CoA. Overexpression of PCC and MCM (Reeves et al., 2007), or CCR (Stassi et al., 1998), increased productions of corresponding acyl-CoA pools. Unfortunately, detailed amino acid metabolisms for the two acyl-CoAs are not well characterized.

1.3 Pikromycin, polyketide antibiotics

1.3.1 Antibacterial mode of action of pikromycin

Pikromycin (PKM) is an antibiotic macrolide produced by *Streptomyces venezuelae*. It is previously reported that PKM has an antibacterial activities by inhibiting growths by repressions of protein synthesis. PKM inhibits gene translation by binding to nascent peptide exit tunnel of 23s rRNA (Almutairi et al., 2017) (Figure 1.7). Similar mechanisms were identified with macrolide type antibiotics such as erythromycin (Ettayebi et al., 1985) and carbomycin (Hansen et al., 2002). PKM, at

the moment, does not have advantages over other macrolide antibiotics. But it is important as a raw materials for productions of clinically important antibiotics (i.e. erythromycin) due to similarities in a polyketide backbone (Kittendorf and Sherman, 2009).

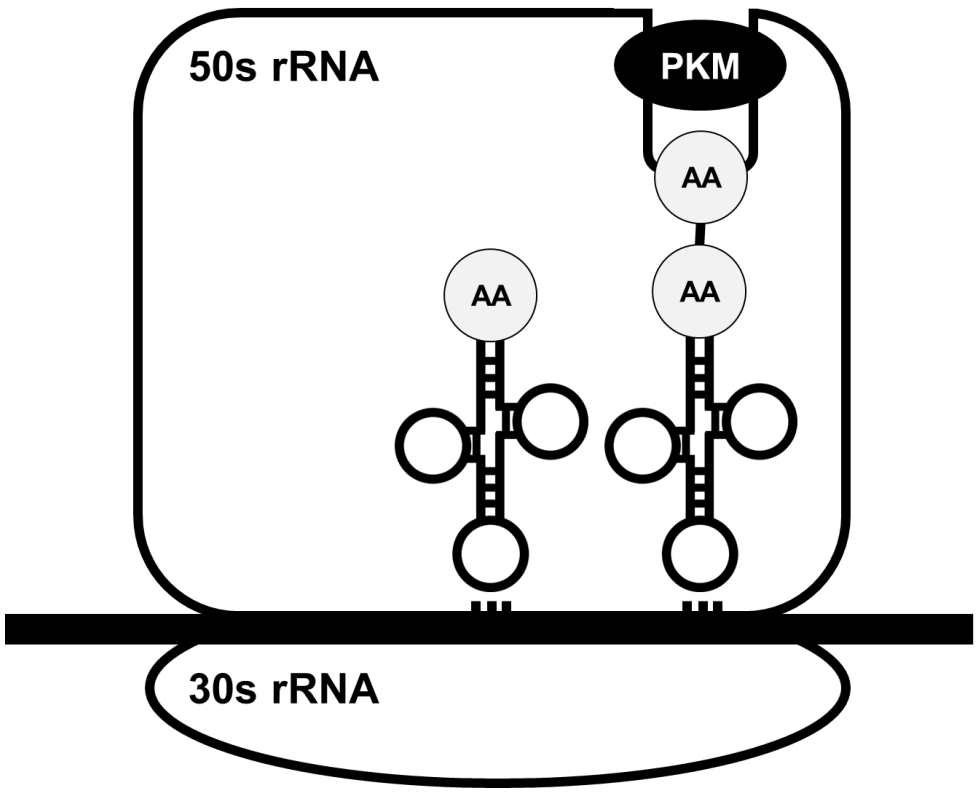


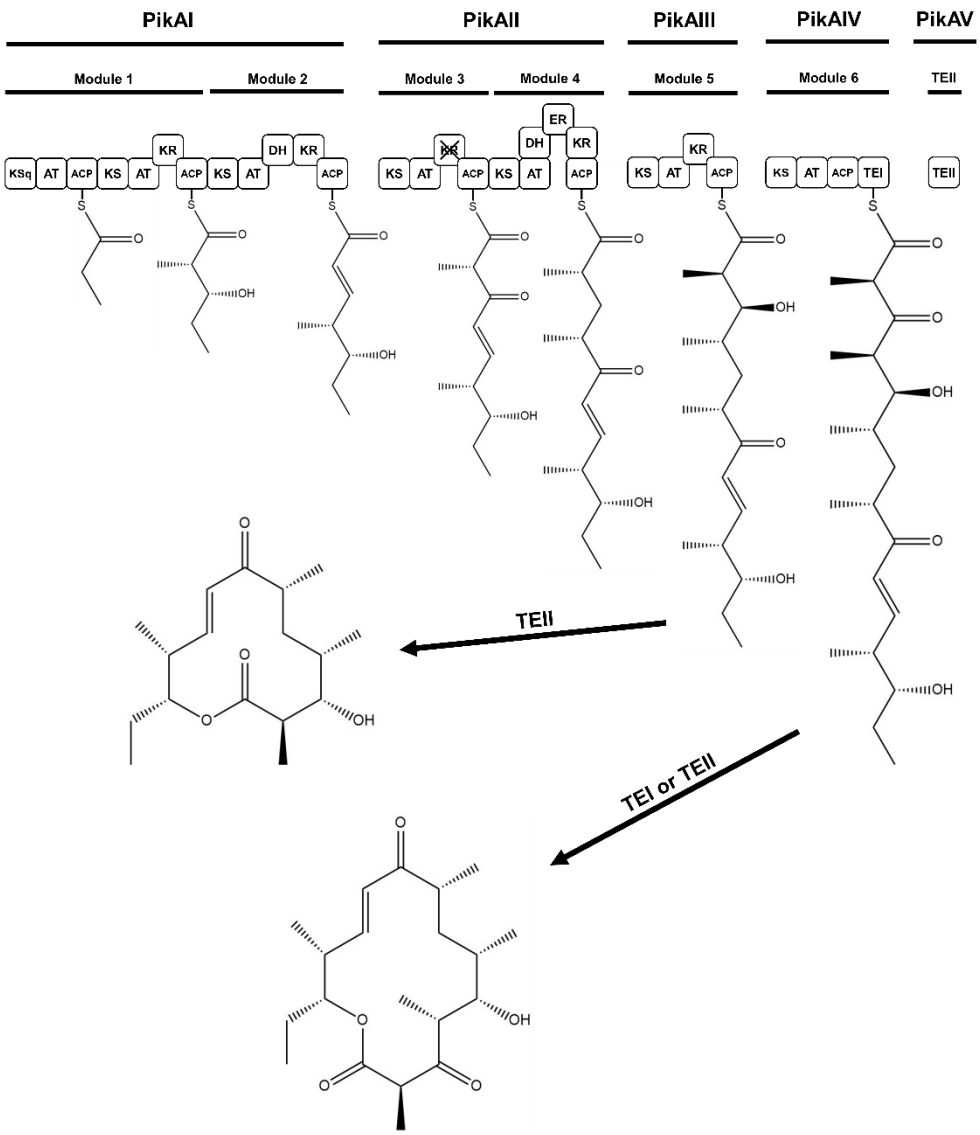
Figure 1.7. Pikromycin Inhibits protein synthesis by blocking peptide exit tunnel of rRNA.

1.3.2 Biosynthesis of pikromycin from *Streptomyces venezuelae*

Pikromycin is produced from type I PKS genes from *S. venezuelae*. It is composed of one malonyl-CoA and six methylmalonyl-CoAs (Wilson et al., 2001; Phelan et al., 2017). A polyketide backbone of PKM is synthesized by five PKS genes, PikAI through PikAV. There are total 6 modules and a TE (Xue and Sherman, 2001).

A unique feature about the PKS genes of PKM is that there are two different types of TE, and the two TE determine synthesis pathway leading to either PKM or methymycin (MTM). TEI, which is a part of module 6, specifically results in narbonolide. But, type II TEII may result in both narbonolide and 10-deoxymethynolide (Kittendorf and Sherman, 2009) (Figure 1.8a). Narbonolide and 10-deoxymethynolide are then glycosylated with desosamine by DesVII, and hydroxylated with PikC, cytochrome P450 monooxygenase (Figure 1.8b).

A


PikAI
PikAII
PikAIII
PikAIV
PikAV
Module 1
Module 2
Module 3
Module 4
Module 5
Module 6
TEII
KSq
AT
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TEI or TEII
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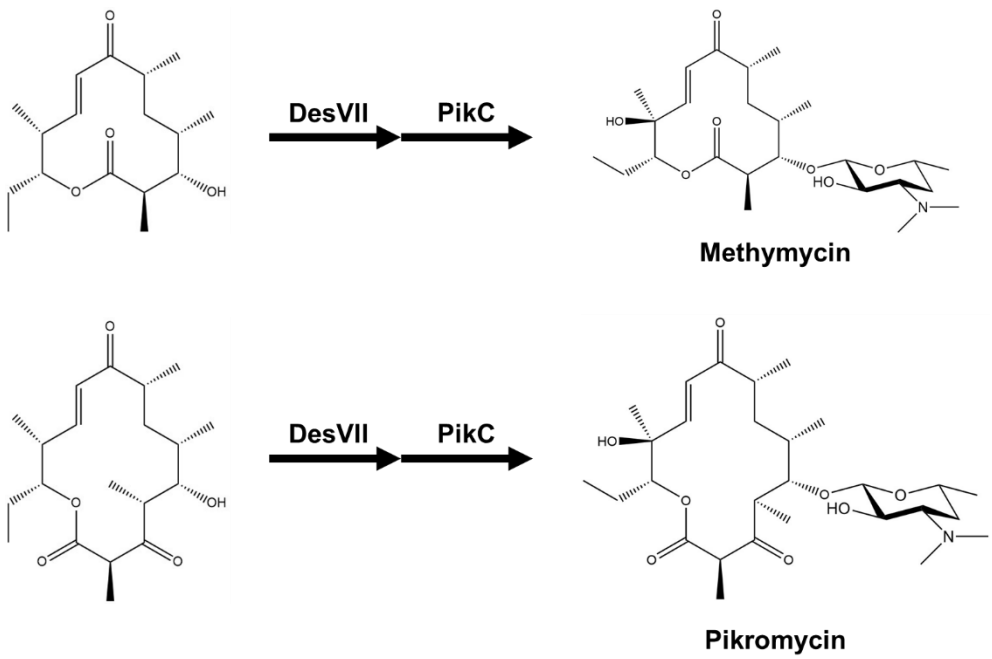


Figure 1.8. Pikromycin and methymycin synthesis pathway. (a) Synthesis of polyketide backbones, and (b) glycosylation and hydroxylation of macrocyclic lactone ring.

1.4 The scope of thesis

The purpose of this thesis is to produce fatty acids and polyketide antibiotics, pikromycin, by utilizing branched chain amino acid catabolism. To attain this end, manipulations of C and N metabolisms, i.e. glycolysis and TCA, and branched chain amino acid metabolism, by controlling expressions of key enzymes in the C and N metabolisms were performed in *Streptomyces coelicolor* and *Streptomyces venezuelae*.

First, synthetic promoters and 5' UTRs were designed and constructed based on multi-omics datasets, ChIP-seq, Ribo-seq, and TSS-seq. There are only small numbers of promoters for gene expressions in *Streptomyces* sp. that it is not as advanced as in *E. coli*. For that reason, using the data sets from next generation sequencing of *S. coelicolor*, 8 plasmids carrying native promoters and 5' UTR of S. *coelicolor* with different transcriptional and translational strengths were constructed.

In chapter 3, BCFA productions were enhanced in *S. coelicolor* for developments of a production host strain for microbial biofuels. Since starter and elongation units of BCFA biosynthesis are shared with that of polyketides, *S. coelicolor* with deletions of PKS gene clusters were used as a host strain. BCAA catabolism and glucose metabolism were investigated for supplementations of acyl-CoA precursors.

In chapter 4, PKM was overproduced in *S. venezuelae* by utilizing C and N metabolisms. BCDH and ACAD were overexpressed for catabolism of BCAA, and MCM was overexpressed to produce methylmalonyl-CoA from central carbon metabolism. A combination of BCDH and MCM expression resulted in the highest

macrolide production, but production of PKM itself was decreased due to activities of *pikC* cytochrome P450.

There still needs more thorough understanding of various acyl-CoA synthesis pathways, and their gene regulations as well. But methodologies and ideas employed in this study provided a glimpse about the relationships of acyl-CoA synthesis pathway to primary and secondary metabolism. Only a couple of acyl-CoA were investigated in this study, but it would provide essential data for elucidating synthesis pathways for other acyl-CoA compounds to produce various secondary metabolites.

Chapter 2.

A Novel Approach for Gene Expression Optimization through Native Promoter and 5' UTR Combinations Based on RNA-seq, Ribo-seq, and TSS-seq of *Streptomyces coelicolor*

A full reprint of the paper published in ACS Synthetic Biology (2017) **6**(3): 555-565.

2.1 Abstract

Streptomycetes are Gram-positive mycelial bacteria, which synthesize a wide range of natural products including over two-thirds of the currently available antibiotics. However, metabolic engineering in *Streptomyces* species to overproduce a vast of natural products are hampered by limited number of genetic tools. Here, two promoters and four 5' UTR sequences showing constant strengths were selected based upon multi-omics datasets from *Streptomyces coelicolor* M145, including RNA-seq, Ribo-seq, and TSS-seq, for controllable transcription and translation. Total eight sets of promoter / 5' UTR combinations, with minimal interferences of promoters on translation, were constructed using the transcription start site information, and evaluated with GusA system. Expression of GusA could be controlled to various strengths in three different media, in a range of 0.03 to 2.4 folds, compared to that of the control, ermE*P / Shine-Dalgarno sequence. This method was applied to engineer three previously reported promoters to enhance gene expressions. The expressions of ActII-ORF4 and MetK were also tuned for actinorhodin overproductions in *S. coelicolor* as examples. In summary, we provide a novel approach and tool for optimizations of gene expressions in *Streptomyces coelicolor*.

Keywords: Promoter engineering, Ribosome Binding Site, Metabolic engineering, *Streptomyces coelicolor*, Antibiotics

2.2 Introduction

Productions of antibiotics from the soil-dwelling microorganism *Streptomyces* (Berdy, 2005) are in great demands for pharmaceutical industries owing to surge of multidrug resistances of bacteria (Pokrovskaya and Baasov, 2010). In order to produce such antibiotics at commercial levels, various genetic tools for fine control of gene expression of biosynthetic pathways and their regulatory network are in great demands (Birch et al., 1993; Coze et al., 2013) due to limited numbers of gene expression systems available for *Streptomyces* sp.. Currently, although other systems such as kasOp and SF14p are available (Wang et al., 2013), ermE* promoter (ermE*P) (Bibb et al., 1985) from *Saccharopolyspora erythraeus* is the most widely used strong and constitutive promoter. Another example is a thiostrepton inducible promoter, tipA, with limited induction levels (Murakami et al., 1989; Takano et al., 1995). As a result, developments of new scalable and predictable gene expression systems are in urgent needs.

To this end, several constitutive promoters were recently evaluated and developed based on the RNA-seq results from *Streptomyces coelicolor* M1146 (Li et al., 2015) and *Streptomyces albus* J1074 (Luo et al., 2015). It is reasonable to construct libraries of promoters with RNA-seq, because the RNA-seq is direct measurement of mRNA transcript levels, and sensitive with broader dynamic range, compared to those from microarray (Marioni et al., 2008). However, RNA-seq data are not enough to design the gene expression system for fine control of gene expression, because actual amounts of protein synthesized depend on the efficiency with which an mRNA is translated, referred as the translation efficiency (TE). TE is

determined by secondary structure of mRNA and ribosome binding site (RBS) affinity for ribosomes (Yin et al., 2015). If TE is low, despite the abundance of mRNA, amounts of protein synthesized are low accordingly (Taylor et al., 2013). Moreover, strengths of RBS along with that of promoters determine protein folding kinetics and solubility (Baneyx and Mujacic, 2004). Protein mis-folding caused by unmatched transcriptional and translational rates of genes may lead to inclusion bodies or nonfunctional proteins (Zhu et al., 2013). There have been many studies to solve such problems in translations, e.g. chaperones (Bukau and Horwich, 1998) and media optimization to comply with codon usage of target protein (perl et al., 1992), but ideally, promoter and RBS combinations should be optimized for the best pairs with predictable and controllable target gene expression.

Along with RNA-seq, quantitative analysis of protein translation is acquired by ribosome profiling (Ribo-seq), which analyzes the ribosome protected mRNA fragments (RPFs) by deep sequencing (Ingolia et al., 2009; Ingolia, 2014). TE is then calculated by dividing the RPF levels by the corresponding mRNA transcript levels for each gene. Accordingly, TE of all available mRNA of a species can be calculated (Gerashchenko et al., 2012). It suggests that we can select native 5' UTR sequences from the pools of thousands of mRNAs with known TE, and design synthetic 5' UTRs to precisely control target gene translation instead of synthetic RBS's.

In this work, a novel method for screening and designing pairs of promoters and 5' UTR regions was suggested considering both transcription and translation. Recent studies mainly searched separately for promoters and RBSs based on RNA-

seq data and 5' UTR designing software, because multi-omics datasets like Ribo-seq and TSS-seq were not available to *Streptomyces* sp.. They are not satisfactory enough to meet our needs to provide strong or optimal gene expression of target genes in actinobacteria. Using TSS information from the very first Ribo-seq and TSS-seq datasets from *S. coelicolor*, promoters and 5' UTRs were constructed so that they would maintain their native TSS, and promoter sequences would not interfere and cross talk with 5' UTR sequences. Total eight sets of promoter / 5' UTR combinations, consisted of two promoters and four 5' UTRs with varying transcriptional and translational strengths, were constructed because *Streptomyces* sp. lack such systems that can control strengths of gene expressions (Murakami et al., 1989; Takano et al., 1995).

The promoters and 5' UTRs were evaluated with β-$_{D}$-glucuronidase (GusA), since GusA assay is proven to be effective in several different *Streptomyces* sp., and is as simple as color assays from β–galactosidase and X-gal (Myronovskyi et al., 2011; Horbal et al., 2014). And then, they were successfully applied to enhance strengths of gene expressions from previously reported promoters for *Streptomyces* sp., $P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$ (Li et al., 2015). The promoter / 5' UTR sets were also applied to differentially overexpress ActII-ORF4 (SCO5085) and MetK (SCO1476) in *S. coelicolor*, two well-known targets with positive effects on the polyketide antibiotic, actinorhodin (ACT), production (Kim et al., 2003; Kim et al., 2011; Wang et al., 2013). In summary, gene expression was successfully predicted and controlled by the selection of promoters and 5' UTRs from multi-omics datasets, i.e. RNA-seq, Ribo-seq, and TSS-seq. This study proposes a new tool, which can be

usefully applied to synthetic biology and metabolic engineering for secondary metabolite productions in *Streptomyces* strains.

2.3 Materials and Methods

2.3.1 Bacterial strains, plasmids, and culture conditions

All the bacterial strains used, and plasmids constructed throughout the study are listed in Table 2.1. Luria-Bertani (LB) broth (Becton Dickinson, USA) was used for *E. coli* strains culture. $R5^-$ complex medium containing 103 g sucrose, 10.12 g $MgCl_2 \cdot 6H_2O$, 10 g glucose, 5.73 g TES buffer (pH 7.2), 5 g yeast extract, 0.25 g K_2SO_4, 0.1 g Difco casamino acids, 7 mL of 1 N NaOH, and 1 mL of a trace element solution (10 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 20 mg $CuCl_2 \cdot 2H_2O$, 20 mg $Na_2B_4O_7 \cdot 10H_2O$, 20 mg $MnCl_2 \cdot 4H_2O$, 80 mg $ZnCl_2$, and 400 mg $FeCl_3 \cdot 6H_2O$ in 1 L distilled water) in 1 L of distilled water was used for culture of *Streptomyces* strains.

Table 2.1. Strains and Plasmids Used in This Study

strains or plasmids	descriptions	references
Streptomyces Strains		
Streptomyces coelicolor A3 (2) M145	wild type (WT) strain	Kim et al., 2012
Streptomyces venezuelae ATCC15439	WT strain	ATCC
Streptomyces avermitilis ATCC31267	WT strain	ATCC
E. coli Strains		
DH5α	a host for general gene manipulations	ThermoFisher
JM110	a strain for demethylation of plasmids	Stratagene
Plasmids		
pIBR25	Amp^R and *tsr* marker; *Streptomyces* vector with SCP2* origin	Thuy et al., 2005
pIBRSDGUS	pIBR25 containing SD RBS sequence and *gusA*, cloned into *Pst*I/*Hin*dIII sites	this work
pP45R51GUS	pIBRGUS containing $P_{4505}R_{5189}$ in *Sac*I/*Pst*I sites instead of P_{ermE*}	this work
pP45R38GUS	pIBRGUS containing $P_{4505}R_{3854}$ in *Sac*I/*Pst*I sites instead of P_{ermE*}	this work
pP45R50GUS	pIBRGUS containing $P_{4505}R_{5061}$ in *Sac*I/*Pst*I sites instead of P_{ermE*}	this work
pP45R57GUS	pIBRGUS containing $P_{4505}R_{5755}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
pP45R06GUS	pIBRGUS containing $P_{4505}R_{0641}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
pP45R30GUS	pIBRGUS containing $P_{4505}R_{3083}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
pP45R20GUS	pIBRGUS containing $P_{4505}R_{2078}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work

pP48R57GUS	pIBRGUS containing $P_{4808}R_{5755}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
pP48R06GUS	pIBRGUS containing $P_{4808}R_{0641}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
pP48R30GUS	pIBRGUS containing $P_{4808}R_{3083}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
pP48R20GUS	pIBRGUS containing $P_{4808}R_{2078}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
$pP_{SCO5768}$GUS	pIBRGUS containing $P_{SCO5768}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
$pP_{SCO5768}$R57GUS	pIBRGUS containing $P_{SCO5768}R_{5755}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
$pP_{SCO4658}$GUS	pIBRGUS containing $P_{SCO4658}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
$pP_{SCO4658}$R57GUS	pIBRGUS containing $P_{SCO4658}R_{5755}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
$pP_{SCO3410}$GUS	pIBRGUS containing $P_{SCO3410}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
$pP_{SCO3410}$R57GUS	pIBRGUS containing $P_{SCO3410}R_{5755}$ in *Bgl*II/*Pst*I sites instead of P_{ermE*}	this work
pIBRactIIorf4	pIBR25 containing *actII-orf4*	this work
pP45R57actIIorf4	pIBRactIIorf4 containing $P_{4505}R_{5755}$ in *Bgl*II/*Xba*I sites instead of P_{ermE*}	this work
pP48R30actIIorf4	pIBRactIIorf4 containing $P_{4808}R_{3083}$ in *Bgl*II/*Xba*I sites instead of P_{ermE*}	this work
pIBRmetK	pIBR25 containing *metK*	this work
pP45R57metK	pIBRmetK containing $P_{4505}R_{5755}$ in *Bgl*II/*Xba*I sites instead of P_{ermE*}	this work
pP48R30metK	pIBRmetK containing $P_{4808}R_{3083}$ in *Bgl*II/*Xba*I sites instead of P_{ermE*}	this work

For evaluations of GusA expressed in different media, YEME, containing yeast extract, malt extract, peptone, and glucose, and SMM, containing glucose and casamino acid as C and N sources, were made by following standard protocols (Kieser et al., 2000).

Appropriate antibiotics, 0.1 mg/mL of ampicillin, 50 µg/mL of apramycin, or 8 µg/mL of thiostrepton, were added to culture broths when necessary. *E. coli* strains were cultured in 37 ℃, and *Streptomyces* strains were cultured in 30 ℃, both with 200 rpm shaking. For *Streptomyces*, 150 mg of wet weighted cell, from initial 50 mL of seed cultures after 24 hr, were inoculated to fresh 50 mL $R5^-$ media.

$R5^-$ agar media ($R5^-$ complex media with 22 g/L agar) were used for solid culture and sporulation of *Streptomyces* strains. The cultures were carried out at 30 ℃ without shaking. Appropriate antibiotics were also added to the agar media when needed.

2.3.2 Strain construction

Streptomyces codon optimized *E. coli* originated *gusA* (JW1609) was PCR amplified using LA-taq DNA polymerase (Takara, Japan) with XbaI cut site containing the primer 1 and HindIII cut site containing primer 2 as tabulated in Table 2.2.

Table 2.2. List of Primers for Promoter and 5' UTR Constructions

primer	sequences
1	AATATCTGCAGATGCTCCGGCCCGTCGAAACCC
2	ATAATAAGCTTTTATCACTGCTTCCCGCCCTG
3	AATTAGATCTCTTAATTAAGTGACCGGCAGCA
4	ATTATAGATCTGGTACCAGCCCGACCCGAG
5	ATATTCTGCAGGTCCGTACCTCCGTTGCTTACCAACCGGCACGATTGTGC
6	ATAATGAGCTCCGACATCACGGGGCCGTCGCA
7	ATTATCTGCAGGGATTCAGGGCCTCCGGGAAGGCCGCTGTT CGCGGTCACATGCTCCGCAGG
8	ATTATCTGCAGGAGGGTCTCTCCTGTCGATGCGCTGCGTGG CGCGGTCACATGCTCCGCAGG
9	ATTATCTGCAGGTTGCGACTTCCCGTACGTGAGATGCGGAG CGCGGTCACATGCTCCGCAGG
10	AATTAGATCTTTCTTGAGGAATTCGACCAGG
11	ATTATAGATCTCGGCACCGGCACCTCGTGGC
12	ATATCTGCAGCGGTGGCTCCCTCCTCGGACCGTGTAACCGCATCCTTCCTT GCTGTGAGCCTAGCACG
13	ATATCTGCAGTGCGTTCTCCTTCGTCGTGTCCGTCGTGTCCGTCGTGTTCC GTGTGTGCGTGCCTCTTGCTGTGAGCCTAGCACG
14	ATATCTGCAGTTCACTCCTCCATGGCCACACGGCGCAGCCTTCTTGCTGT GAGCCTAGCACG
15	GTGCCCTCACGGAAGTGTCTTGCTGTGAGCCTAGCACG
16	CGTGCTAGGCTCACAGCAAGACACTTCCGTGAGGGCAC
17	GTGCCCTCACGGAAGTGTGTCCGTCTCGCAGGTTATCG
18	TAACCTGCGAGACGGACACACTTCCGTGAGGGCAC

19	ATATCTGCAGGACCTGTGCTTCCCTCTCC
20	ATTATAGATCTAGGACTCGGGGCCGTAGACCT
21	ATATTCTGCAGGTGCGTCCCCTCTCCGACCT
22	ATTATAGATCTTAGAGCGGGAAGAACGCGACCGA
23	ATATTCTGCAGCGCGCCTCCCTCCTCATGAC
24	ATTATAGATCTTGGACCGCCGCCTGTTCACCT
25	ATATTCTGCAGACCCTCCTGTTGACCTCAGTATGGTCA
26	ATATAGATCTCCTGTTCACCTCGACCG
27	ATATCTGCAGCGGTGGCTCCCTCCTCGGACCGTGTAACCGCATCCTTCCT CAGTATGGTCACTCAA
28	ATATAGATCTGGAAGAACGCGACCGAGTTC
29	ATATCTGCAGCGGTGGCTCCCTCCTCGGACCGTGTAACCGCATCCTTCTT GTGCGATCTTATGGCAG
30	ATATAGATCTCCGTAGACCTCCACGACAC
31	ATATCTGCAGCGGTGGCTCCCTCCTCGGACCGTGTAACCGCATCCTTCCA TCCGCATCCAACCACCT
32	ATATTCTAGAATGAGATTCAACTTATTGGGAC
33	AATTAAGCTTCTACACGAGCACCTTCTCAC
34	ATATTTCTAGAATGTCCCGTCGCCTGTTCACCTC
35	ATAATAAGCTTATCCTTACAGGCCCGCGGCCT
36	ATATAAGCTTGGCGTAATCATGGTC

It was cloned into XbaI/HindIII (Thermo, USA) sites of pIBR25 vector to give pIBRGUS. Since *gusA* contained SacI cut site, it was necessary to replace SacI cut site with that of BglII to replace ermE*P. pIBRGUS was PCR amplified using the primer 1 and BglII cut site containing the primer 3. The PCR fragment containing pIBRGUS sequences without ermE*P was ligated with fragments of target promoter and RBS combinations.

For a reference, ermE*P was PCR amplified with the primer 4, and SD sequence containing the primer 5, and inserted into pIBRGUS to give pIBRSDGUS. To construct P_{4505} with R_{5189}, R_{3854}, and R_{5061} in conventional method, the primers 6 and 7-9 were used before GusA was inserted into the plasmid. P_{4505}, and P_{4808} were PCR amplified from TSS to -300 of each SCO4505 and SCO4808 with the primer 10 and 12-14, and the primer 11 and 12-14, respectively. Each primer sets contained the three 5' UTR sequences, R_{5755}, R_{0641}, and R_{3083}, all from TSS to -1 of start codon, in reverse primers. R_{2078} was inserted by fusion using the primer sets, 15, 16, and 5 for $P_{4505}R_{2078}$, and 17-19 for $P_{4808}R_{2078}$.

Promoters of SCO5768, SCO4658, and SCO3410 were PCR amplified with primer sets 20 and 21, 22 and 23, and 24 and 25, respectively, and the PCR amplified fragments were inserted into pIBRGUS to give the three plasmids, $pP_{SCO5768}$GUS, $pP_{SCO4658}$GUS, and $pP_{SCO3410}$GUS. To construct $pP_{SCO5768}$R57GUS, $pP_{SCO4658}$R57GUS, and $pP_{SCO3410}$R57GUS, SCO5768, SCO4658, and SCO3410 promoters were PCR amplified using primer sets 26 and 27, 28 and 29, and 30 and 31, respectively.

actII-orf4 (SCO5085) and *metK* (SCO1476) of *S. coelicolor* were individually cloned into XbaI/HindIII sites of the promoter sets using the primers sets 32 and 33, and 34 and 35, respectively. For ermE*P expressed *actII-orf4*, the primer 33 and 36 were used.

E. coli DH5α was used for multiplication of plasmids, and *E. coli* JM110 was used to acquire non-methylated DNA plasmids. Standard procedures were used to introduce the non-methylated plasmids into *S. coelicolor*, *S. venezuelae, and S. avermitilis* (Kieser et al., 2000).

2.3.3 RNA-seq, Ribo-seq, and TSS-seq data

Multi-omics datasets used throughout the study are from the previously published study (Jeong et al., 2016), where raw data were deposited in the Gene Expression Omnibus archive with accession code GSE69350. It includes ssRNA-seq and Ribo-seq data, acquired at mid-exponential phase, transition phase, late exponential phase, and stationary phase, and dRNA-seq data.

2.3.4 GusA activity measurements of the promoter / 5' UTR combinations

On day 4 of 50 mL cell cultures, 2 mL sample was harvested for GusA activity measurement. After removal of supernatants with centrifugation at 13500 rpm for 2 min, cell pellets were reconstituted and underwent lysis with 1 mL of lysis buffer containing, 50 mM phosphate buffer with pH 7.0, 5 mM dithiothreitol (DTT), 0.1%

Triton-X, and 1 mg/mL lysozyme, at 37 ºC for 15 min. 5 µL of a 0.2 M substrate, *p*-nitrophenyl-β-$_D$-glucuronide, was added to 0.5 mL of 8 folds diluted lysates for P_{4808} sets. And the other 0.5 mL of the lysates without the substrate was diluted by 8 folds, and control was used to normalize the data. Reaction was carried out at 37 ℃ for 5 min, and 0.5 mL of Na_2CO_3 was added to terminate the reaction. UV absorbance at 415 nm was measured with Multiskan spectrum (Thermo scientific, USA) (Wang et al., 2014). Values of the UV absorption, normalized by the controls, was divided by reaction time (min) multiplied with dried cell weight of used cells (mg) to calculated Gus Unit (GU) [A415/(min·mg)]. Assay data were averaged out with triplicate experimental values.

2.3.5 Actinorhodin measurements

To measure ACT production of each mutant, 1 mL of culture sample was harvested every day. 4N KOH solution was added to the harvested sample, final 1N KOH concentrations, followed by 200 rpm shaking at room temperature for 5 min. The supernatant was acquired by centrifugation at 13500 rpm for 5 min at room temperature. Using Multiskan spectrum (Thermo scientific, USA), absorbance at 640 nm of the supernatant was measured (Ochi and Freese, 1982)

2.4 Results and Discussion

2.4.1 Promoter and 5' UTR target selections based on Multi-omic data

RNA-seq and Ribo-seq data acquired at 4 different growth phases from R5$^-$ complex medium cultures, and TSS-seq data measured under various growth conditions, in our previous report (Jeong et al., 2016), were used to select promoters and 5' UTRs from *S. coelicolor* genome. The data were obtained from Gene Expression Omnibus (GEO) database using accession number of GSE69350. The multi-omics datasets could explain important biological features about *S. coelicolor* in a great detail. It included TSS information, such as numbers of leaderless genes, and lengths of 5' UTR sequences. Various aspects of transcription and translation are also studied, which included information such as transcriptional patterns, correlations between transcription and translation, transition of metabolism from primary to secondary, etc. The datasets comprised of such comprehensive information, which enabled us to make meaningful selections and constructions of promoters and 5' UTRs.

Genes having both constitutive transcription and stable translation were targeted for selection of promoters and 5' UTR candidates (Figure 2.1). Reads per Kilobase per Million mapped reads (RPKM) values, calculated by total reads of a gene divided both by its length in kilobase and total million mapped reads of the genome, which represent quantitative transcription level of the genes, varied greatly from 0 to 45000. Among them, genes with RPKM values under 600 were eliminated as they can be seen as under-expressed.

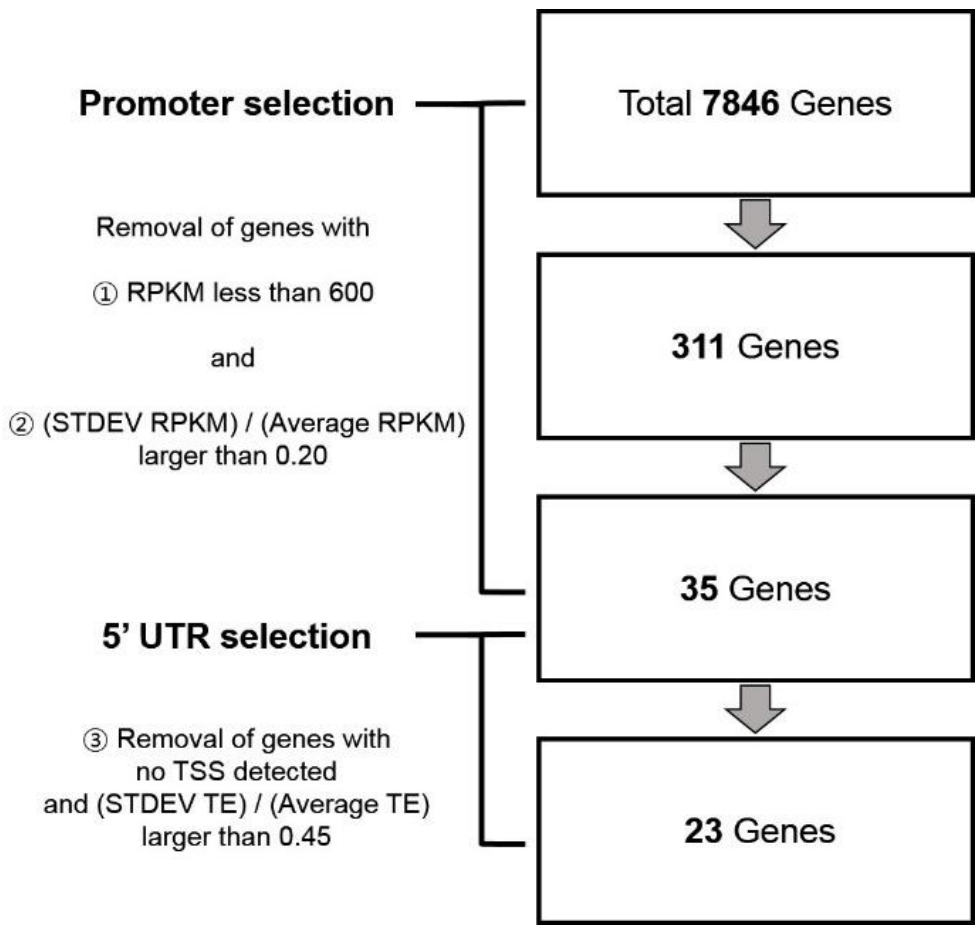


Figure 2.1. Scheme for selection of target promoter and 5' UTR genes from RNA-seq, Ribo-seq, and TSS-seq.

At this stage, the size of candidate pool was reduced down to 311 genes out of 7846 genes. Among them, only the genes with their RPKM level changes less than 20%, or 0.2 folds, of their average, were considered to be constitutive. The cutoff values for the promoter selections were up to 10 folds stricter than other previous reports (Li et al., 2015; Luo et al., 2015). In this way, our promoter candidates were more reliable for constitutive transcription due to the datasets acquired at more growth time points, and very thorough and harsh cutoff conditions. There are no limits to the selection of promoter targets. For example, housekeeping genes such as *hrdB* can be a good candidate for promoters. But according to our study, *hrdB* gene did not belong to our 35 candidates. *hrdB* had an average RPKM value of 513 ± 172, where the fluctuation was about 34% (0.34 folds) of the average. Resulting 35 genes were further screened for 5' UTR targets.

TE varied greatly from 0.20 to 29.09, with an average of 1.09 for the entire genes. TE values may fluctuate depending on various conditions such as growth phases (Gingold and Pilpel, 2011). Thus, TE should also be as constitutive as possible throughout the life cycle of the cells, for predictable and stable protein expression. The genes with their TE value changes over 45%, calculated by standard deviation of TE divided by the average TE value throughout the four time point sets, were removed from the target list. The genes without definite transcription start site, such as leaderless or undetected based on TSS-seq, were removed at this point. Final 23 genes were selected, which are listed in Table 2.3 and in Table 2.4 for their RPKM and TE values at different growth phases.

Table 2.3. List of 23 selected targets for promoter and 5' UTRs

	Gene
SCO0247	Hypothetical protein
SCO0641	Tellurium resistance protein
SCO1478	RNA polymerase
SCO1489	DNA binding protein
SCO1517	Putative secreted protein
SCO1936	Transaldolase
SCO2078	Putative mebmbrane protein
SCO2082	Cell division protein
SCO2314	Possible integral membrane protein
SCO2617	ATP-dependent Clp protease
SCO3083	Possible integral membrane protein
SCO3187	Hypothetical protein
SCO3404	Cell division protease
SCO3409	Putative inorganic pyrophosphatase
SCO4505	Cold shock protein
SCO4662	Elongation factor
SCO4808	Suc-CoA synthetase beta subunit
SCO4809	Suc-CoA synthetase alpha subunit
SCO5357	Transcription termination factor
SCO5627	Ribosome recycling factor
SCO5755	Possible transcriptional regulator
SCO6467	Phosphatidylserine synthase
SCO6468	Phosphatidylserine decarboxylase

Table 2.4. List of RPKM and TE Values of the Selected 23 Targets

	RPKM				TE			
	EE[a]	ME[b]	LE[c]	S[d]	EE[a]	ME[b]	LE[c]	S[d]
SCO0247	895	999	840	851	1.43	0.90	0.77	0.96
SCO0641	**1974**	**2539**	**2688**	**2244**	**2.52**	**1.44**	**1.64**	**1.76**
SCO1478	2051	1672	1623	1890	0.73	0.60	0.83	0.90
SCO1489	957	804	978	746	0.90	0.72	0.63	0.91
SCO1517	745	718	680	499	2.21	1.06	1.82	1.34
SCO1936	958	1125	779	1063	1.52	1.53	1.78	0.90
SCO2078	**1911**	**1894**	**1771**	**2482**	**0.35**	**0.69**	**0.56**	**0.44**
SCO2082	600	709	588	670	1.05	1.11	1.11	0.88
SCO2314	722	765	667	565	1.13	0.54	0.79	0.85
SCO2617	641	753	616	624	0.54	0.53	0.84	0.51
SCO3083	**1781**	**2407**	**2370**	**2204**	**1.15**	**1.25**	**1.30**	**1.02**
SCO3187	785	741	767	519	2.67	1.43	1.81	1.95
SCO3404	958	820	885	755	0.97	0.90	1.00	0.49
SCO3409	1547	1553	1383	1085	2.11	1.12	1.36	1.15
SCO4505	**25523**	**19949**	**18461**	**16299**	**0.65**	**0.48**	**1.07**	**1.34**
SCO4662	10664	9165	9088	7396	2.94	1.75	1.66	1.47
SCO4808	**2942**	**2723**	**2000**	**2023**	**1.90**	**1.60**	**1.18**	**0.94**
SCO4809	2877	2508	1979	1924	2.03	1.76	1.36	1.03
SCO5357	593	560	549	721	1.24	0.62	0.73	0.62
SCO5627	1092	1135	979	980	1.01	0.53	0.91	0.73
SCO5755	**586**	**638**	**563**	**630**	**2.28**	**6.85**	**7.53**	**5.85**
SCO6467	1133	1056	812	950	0.40	0.23	0.23	0.20
SCO6468	973	861	740	977	0.66	0.48	0.48	0.35

a. EE: Early exponential phase
b. ME: Mid exponential phase
c. LE: Late exponential phase
d. S: Stationary phase

Total two promoters and four 5' UTR were selected from the 23 candidates. There are so many possible combinations of promoters and 5' UTRs to test, even from 23 selected candidate genes. We selected promoters and 5' UTR that have definitive differences in their gene expression because it is hard to distinguish the effects if target combinations have small differences in RPKM and TE. Cold shock protein *scoF2* (SCO4505, P_{4505}), the strongest of the 23 candidates, and succinyl-CoA synthetase *sucC* (SCO4808, P_{4808}), showing 1/10 strength of *scoF2*, were chosen as strong and weak promoters, respectively. A putative transcriptional regulator *clgR* (SCO5755, R_{5755}) with the largest TE was selected as the 5' UTR with the highest TE value. For 5' UTR with smaller TE values, *terD* (SCO0641, R_{0641}), and two putative proteins, SCO3083 (R_{3083}) and SCO2078 (R_{2078}), were selected as shown in Table 2.5, and their specific sequences are listed in Table 2.6. In this way, the eight combinations could represent most of the constitutive promoters and 5' UTRs.

Table 2.5. List of genes for the promoter / 5' UTR constructions

	Gene	Average RPKM	Average TE	TSS
Promoter				
SCO4505	Cold shock protein, *scoF2*	20058	0.88	4926107
SCO4808	Succinyl-CoA synthetase beta subunit, *sucC*	2422	1.40	5234868
5' UTR				
SCO5755	Possible transcriptional regulator, *clgR*	604	5.63	6293028
SCO0641	Tellurium resistance protein, *terD*	2361	1.84	681905
SCO3083	Putative integral membrane protein	2190	1.18	3377207
SCO2078	Putative membrane protein	2015	0.51	2232419
Random 5' UTR (without TSS considerations)				
SCO5189	Hypothetical protein	791	14.32	5647134
SCO3854	Septation inhibitor protein, *crgA*	343	3.49	4239443
SCO5061	GTP-dependent nucleic acid-binding protein, *engD*	236	1.39	Undetected

Table 2.6. Nucleotide Sequences of Promoters and 5' UTRs

	Sequence (5' to 3')
Promoter	
SCO4505	TTCTTGAGGAATTCGACCAGGGTGACGGGACCGATGTCGAGAT CACCGCGCACCAGCTGCTCGCTGAGCTTCTCCGGGGTGTCCTTC GTCAGCTCGAAGTCGAGGAGCGTGCCTGTTCTCGCGAGCCCCCA GTACAGGGGCAGGCAGTTCAGGAACTGGATGTGGCCGACACGC GGCCGGGTGCGAGAATTGTCCACATCGCGAGGCTAGCCCTCCC CCGGGAACCGCCCCTCGCCGACCCCCGGCGTCAGCCGAACGGC GCAGGTGTTCAAACGTCCGGGTGAAGTGATCTTGACCTCTGTTG CGCTCGGGGGCCTGCGTGCTAGGCTCACAGC
SCO4808	CAGACCCCGCGGGGCGGCAGGCCCCGGACGGCCGAAAGCCGTC GGCCTCCGTCCGGCCGAAACCCCGCGGTCCGGTCCGGCGGGTC CCCATTGGGGTGGGGACCAGCCGGACCGCACCCCGGCGGACCG CATCCGTGCGGCCCGCCGGGAGCCCACGGGCCTCACCGGGTCA ACCCCAGCCCGGTGAGGCCCTTCACCCGATCTGCGGAGCGCCCC GTGTCCACCTCACGCACGGCACCGCGTGCCAGTGAGCGCGGCA TCATGTGACAGGCATCACCGCTCAGGTGTGACCCACGATTTAGA GACCCCCGGGAAGCGGCGATAACCTGCGAGAC
5' UTR containing RBS	
SCO5755	GAAGGATGCGGTTACACGGTCCGAGGAGGGAGCCACCG
SCO0641	AGGCACGCACACACGGAACACGACGGACACGACGGACACGAC GAAGGAGAACGCA
SCO3083	AAGGCTGCGCCGTGTGGCCATGGAGGAGTGAA
SCO2078	ACACTTCCGTGAGGGCACTCCGGTGATCATGAATCTCACTGAGA TGGATGACACAGATGCGAAGCGACTTGTCGACTTTGCGGCCGG TTTGGTGTTTGGTCTTCACGGCAGTATCGAGCGGGTGACGCAGA AGGTGTTCCTGCTGTCTCCTGCTAACGTCGATGTCACGGCGGAG GACAAGGCCCGTATCGCAGAGGGCGGGTTCTTCAACCAGAGCT GAGACGCACTACCGGTACAGAGCAAGAACACGGGCCCGGGAC AGGGCCCGAGAGATGGTTCAGGGGAGAGGGAAGCACAGGTC

2.4.2 Construction and validation of promoters and 5’ UTRs

TE is highly dependent on secondary structures of mRNA, so that there should be minimized interferences from its promoter sequences or terminal sequences of upstream genes, e.g. where transcription starts or where ribosomes bind, to maintain stable and observed TE values (Bashor and Collins, 2012). Thus, translation rates of the 23 candidate genes, including the four selected 5’ UTRs (SCO5755, SCO0641, SCO3083, and SCO2078), were predicted beforehand with RBS calculator (Salis et al., 2009) for the guidance of designing 5’ UTR regions. The resulting calculations were compared with experimental TE values from Ribo-seq (Jeong et al., 2016). Unfortunately, however, there were no correlation between the predicted translational strengths and experimentally acquired TE values, owing to a couple of reasons. (Figure 2.2)

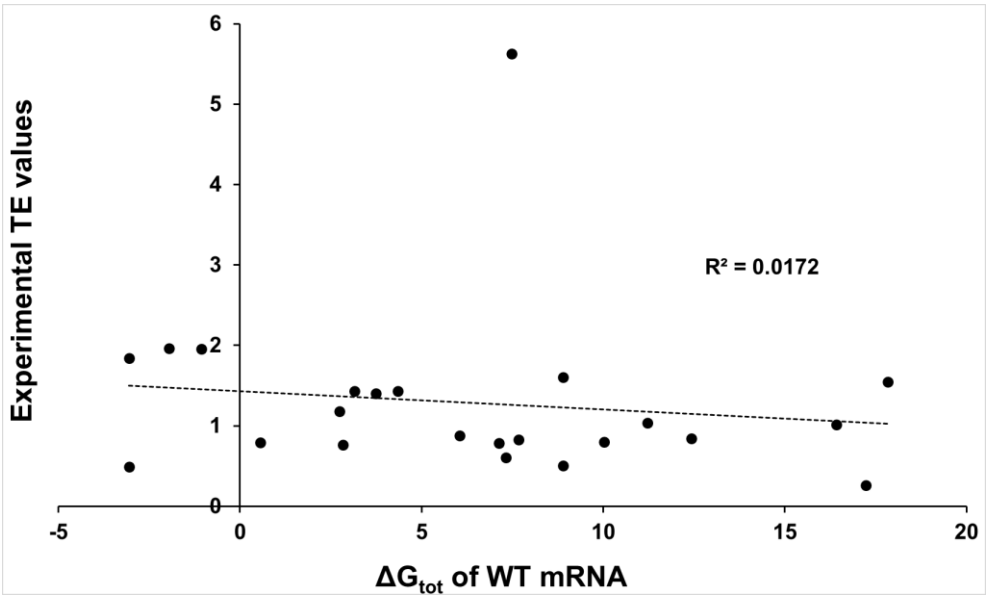


Figure 2.2. Comparison of translational efficiency and Gibbs free energy calculated from RBS calculator. There are no clear correlations between predicted ΔG_{tot} and experimental TE values.

RBS calculator predicts the translational strength by calculating changes in Gibbs free energy, ΔG_{total}, of mRNA with 5 different variables, which are ΔG's of i) mRNA-rRNA interactions, ii) start codon – initiating tRNA anticodon loop hybridization, iii) unfolding mRNA subsequence, iv) unfolding standby site, and v) ΔG loss due to the space between 16S rRNA binding site and start codon. Of all, the unfolding kinetics of standby site is considered as the most important factors affecting translation rate (Espah et al., 2014), because there are high possibility of having increased numbers of the standby sites as the lengths of 5' UTR region become longer. *S. coelicolor* mRNAs often contain very wide ranges of 5' UTR lengths, from leaderless to over 456 nucleotides long. Determined by multi-omics datasets, 413 transcripts from total 2705 known coding sequences were found to be longer than 150 nucleotides (Jeong et al., 2016). It was longer than RBS calculator and / or UTR designer (Seo et al., 2013) could make a plausible predictions. Thus experimental TE values were more likely to be different from the predictions made. In addition, 5' UTR designing methods were specific to open reading frames. As a result, designing our four 5' UTRs was done following a different method. Instead of designing 5' UTR region based on modeling, complete 5' UTR sequences of the four 5' UTR targets were cloned. (Figure 2.3)

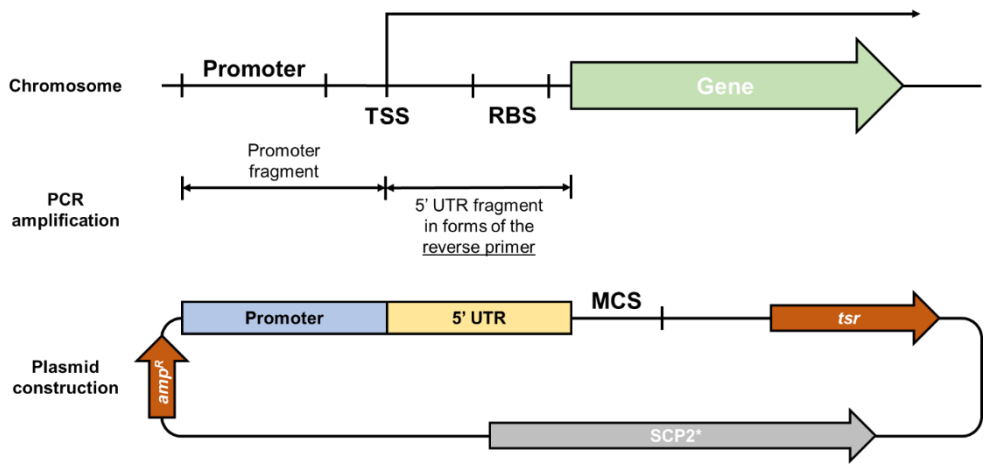


Figure 2.3. Design of promoter / 5' UTR fragment construction. 5' UTR fragments were synthesized into reverse primers for PCR amplification of promoter targets. The PCR fragments were then inserted into pIBR25 in place of ermE*P.

One of the previous studies on promoter and RBS engineering used an insulator between a promoter and RBS, such as RIboJ, to minimize promoter sequences interfering with mRNA sequences structures, and altering TE (Bai et al., 2015). Instead of insulators, promoters and 5' UTRs were designed to have their natural TSS in this study. For example, $P_{4505}R_{5755}$ would start its transcription on the same nucleotide as that of wild type 5' UTR of SCO5755. In this way, the eight promoter / 5' UTR combinations would have the same 5' UTR structures with WT transcripts from the four 5' UTR targets. As a result, Gibbs free energy of mRNA unfolding would become the only variable altering TE from the experimental TE values. The mRNA unfolding energy is also fixed to the gene coding sequences. This makes the eight promoter / 5' UTR be universally applicable to optimize gene expression independently, without the needs of designing gene specific 5' UTRs.

To validate whether or not the constructed promoter / 5' UTR sets are functional, evaluation was carried out with GusA assay system (Myronovskyi et al., 2011; Horbal et al., 2014). Because most of *Streptomyces* species have their own native catechol 2, 3-monooxygenase (*xylE*) gene, such as SAV1615 from *Streptomyces avermitilis*, assays with *xylE* show higher background noises with less sensitivities than that of GusA. For the same reason, GusA system was also more sensitive to the enhanced green fluorescent protein reporter system for the measurement of promoter strengths (Hornal et al., 2014). The expression systems constructed following a conventional cloning method were also evaluated to examine effectiveness of the eight promoter / 5' UTR combinations constructed with TSS information. GusA was expressed with the strong P_{4505} promoter that included

three randomly chosen RBS sequences, in the order of R_{5189}, R_{3854}, and R_{5061} with a decreasing order of TE. About 30 bp upstream regions of the randomly chosen genes were inserted without considering their TSSs.

Examining ermE*P and the four 5' UTR combinations were unconsidered due to unavailable RPKM value of ermE* promoter, and uncertainty of TSS in pIBR25 plasmid. Even though ermE*P is one of the most widely used promoter, it is not completely characterized (Wang et al., 2013). Based on a sequencing data of ermE* promoter in pIBR25 plasmid (Figure 2.4), it was composed of two ermE* sequences, complete sequences of ermE2 and partial sequences of ermE1.

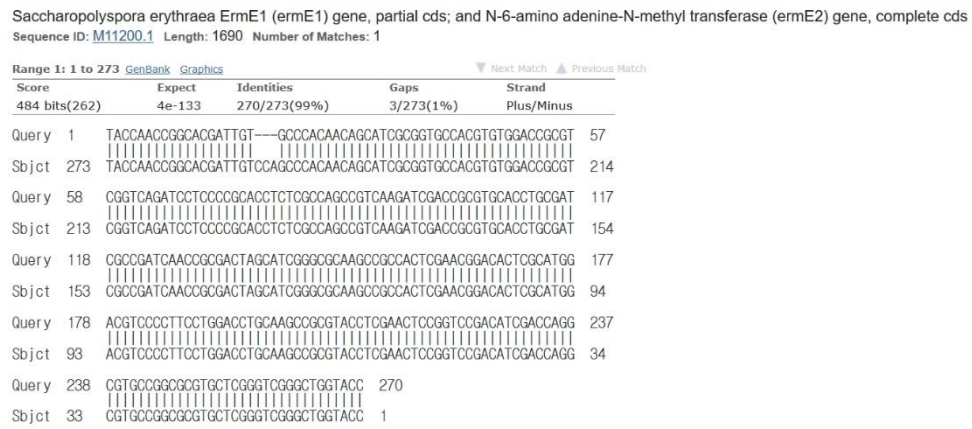
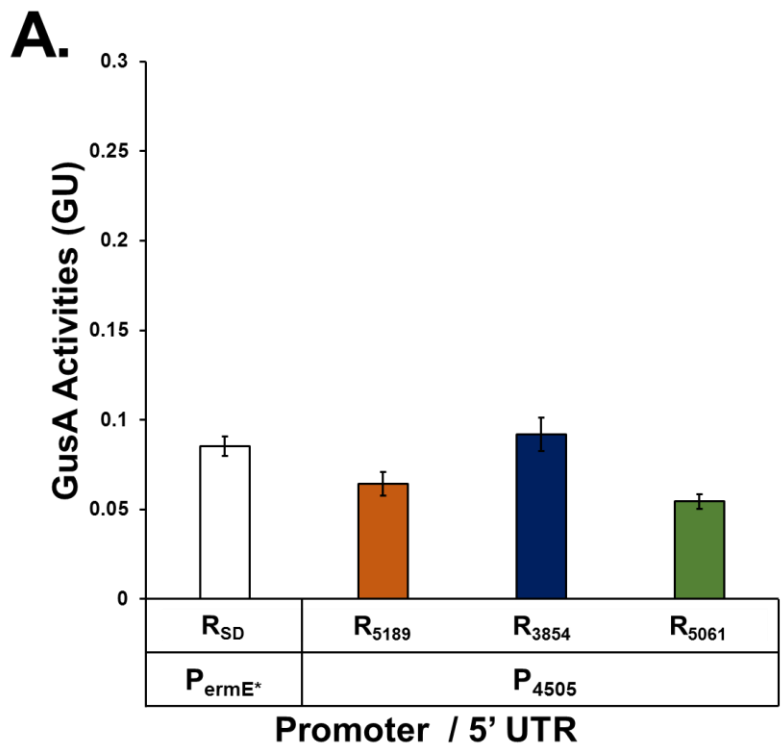


Figure 2.4. Sequence alignment of ermE*P of the WT strain, and ermE*P in pIBR25 plasmid. ermE*P of pIBR25 plasmid contains multiple ermE*P sequences including RBS.

As a result, accurate evaluation of 5' UTRs were not possible because TSS information of ermE promoter in pIBR25 was not available. TSS information was very important in the constructions of 5' UTRs in this study. But as a reference, GusA expressed with *Streptomyces* Shine-Dalgarno (R_{SD}) sequence (AGCAACGGAGGTACGGAC) (Herai et al., 2004) containing ermE* promoter was used. Strengths of the eight promoter / 5' UTR combinations were ranked based on the RPKM and TE values, such as stronger GusA expression with increasing order of (RPKM * TE) values.

Despite the differences in the observed values of TE from Ribo-seq data, when 5' UTRs were constructed with conventional methods, there were no correlations between their TE values and GusA activities (Figure 2.5a). All the three expression levels were about the same as that of the control, P_{ermE*} / R_{SD}. On the other hand, when the expression systems were constructed in consideration of TSS, GusA assay results matched well with the predicted orders of the combinations (Figure 2.5b). The four 5' UTRs were functional as predicted, indicating that there were minimal interference of promoter sequences on translations. If there were cross-talks between the promoters and 5' UTRs, GusA expression will not comply with predicted GusA activities, when a promoter was changed from P_{4505} to P_{4808} for the four 5' UTRs.


A.
GusA Activities (GU)
0.3
0.25
0.2
0.15
0.1
0.05
0
R_SD
R_5189
R_3854
R_5061
P_ermE*
P_4505
Promoter / 5' UTR

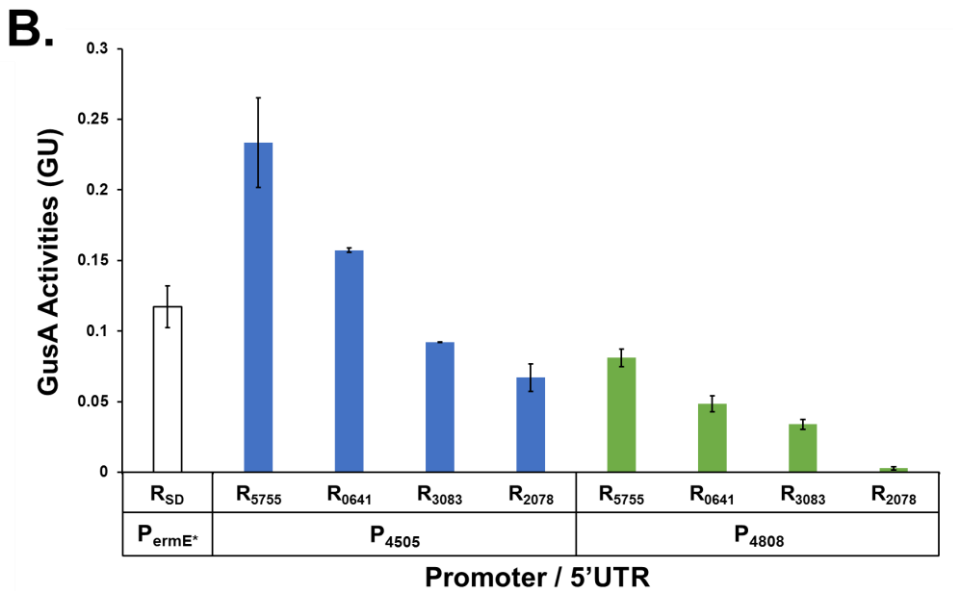


Figure 2.5. GusA evaluations from cell lysates of *Streptomyces coelicolor* M145 strains, having $P_{ermE}R_{SD}$ as a reference. (a) GusA assays of the reference and P_{4505} expression with three randomly chosen, and conventionally constructed RBS sequences. (b) GusA assay of the eight promoter / 5' UTR combinations. The results are first, in order of decreasing promoter strength, and second, in order or decreasing TE.

This result confirmed that our design method could be successfully manipulated according to the order of the prediction made by (RPKM * TE) values. In addition, when expressed by the two strongest promoter / 5' UTR combinations, $P_{4505}R_{5755}$ and $P_{4505}R_{0641}$ respectively, GusA expression could be increased by about 2 folds and 1.3 folds compared to that of P_{ermE*} / R_{SD}. GusA gene expression could also be manipulated in a range from 0.03 to 2.4 folds of ermE* promoter and SD sequence expression.

GusA expressions in cultures from different media were also evaluated to examine whether the applications were limited to $R5^-$ complex media or not. YEME and Supplemented Minimal Media (SMM) were selected for their varying constituents. Expressions of GusA in YEME media were almost identical to those from $R5^-$ complex media, with two exceptions (Figure 2.6a). GusA expressions from both $P_{4505}R_{5755}$ and $P_{4505}R_{0641}$ were increased by about 3 folds, which correspond to about 8.8 folds and 4.5 folds stronger than that of P_{ermE*} / R_{SD} respectively. Gene expressions from the eight promoter / 5' UTR sets covered about 0.16 to 8.8 folds of the control. In the case of SMM, overall gene expressions including P_{ermE*} / R_{SD} were about halved. The eight promoter / 5' UTR sets covered gene expressions from about 0.10 folds to 6.4 folds of P_{ermE*} / R_{SD} expression (Figure 2.6b).

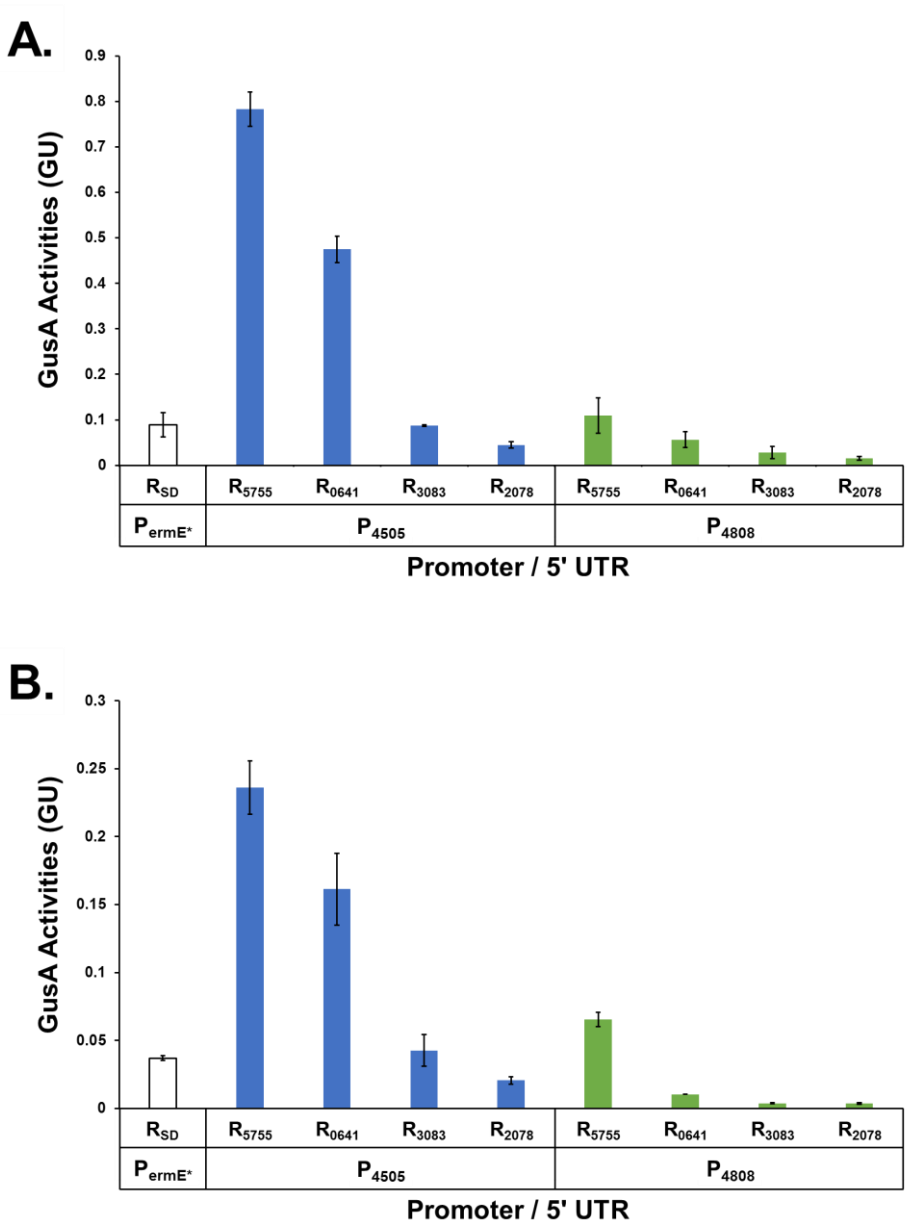


Figure 2.6. **GusA evaluations from *S. coelicolor* strains carrying the eight promoter / 5' UTR expression systems.** They were grown in (a) YEME and (b) SMM. Expression of GusA could be successfully manipulated both in YEME media and SMM, just as in that from R5$^-$ complex media.

In both cases, overall gene expressions were different from those in R5$^-$ media, but the eight combinations were fully functional, and could express GusA in various degrees.

Although the GusA expression of $P_{4505}R_{5755}$ was increased by at least about 2.4 to 8.8 times compared to that of the control in various conditions, the fold changes in the expression levels were not as high as the (RPKM * TE) values predicted. However, the expressions from all the three different media are proximally close to the prediction, and the eight promoter / 5' UTR systems were shown to be functional for optimizing gene expressions with varying strengths. Again, since a different gene was translated from the given four 5' UTRs, their expression levels could be altered to a certain extent. The other explanation would be competitions of mRNAs for ribosomes, and resulting changes in overall translatome. There could be other possible explanations such as mRNAs competing for ribosomes (Mehra and Hatzimanikatis, 2006) or tRNA availability (Varenne et al., 1984), but GusA was successfully expressed with different levels following predicted orders.

To examine whether the promoters and 5' UTRs were functional in other strains as well, the eight plasmids carrying promoters / 5' UTRs and *gusA* gene were transformed into *S. venezuelae* and *S. avermitilis*. While 5' UTRs seemed to be as functional as in *S. coelicolor*, P_{4505} was not functional in both *S. venezuelae* (Figure 2.7a) and *S. avermitilis* (Figure 2.7b).

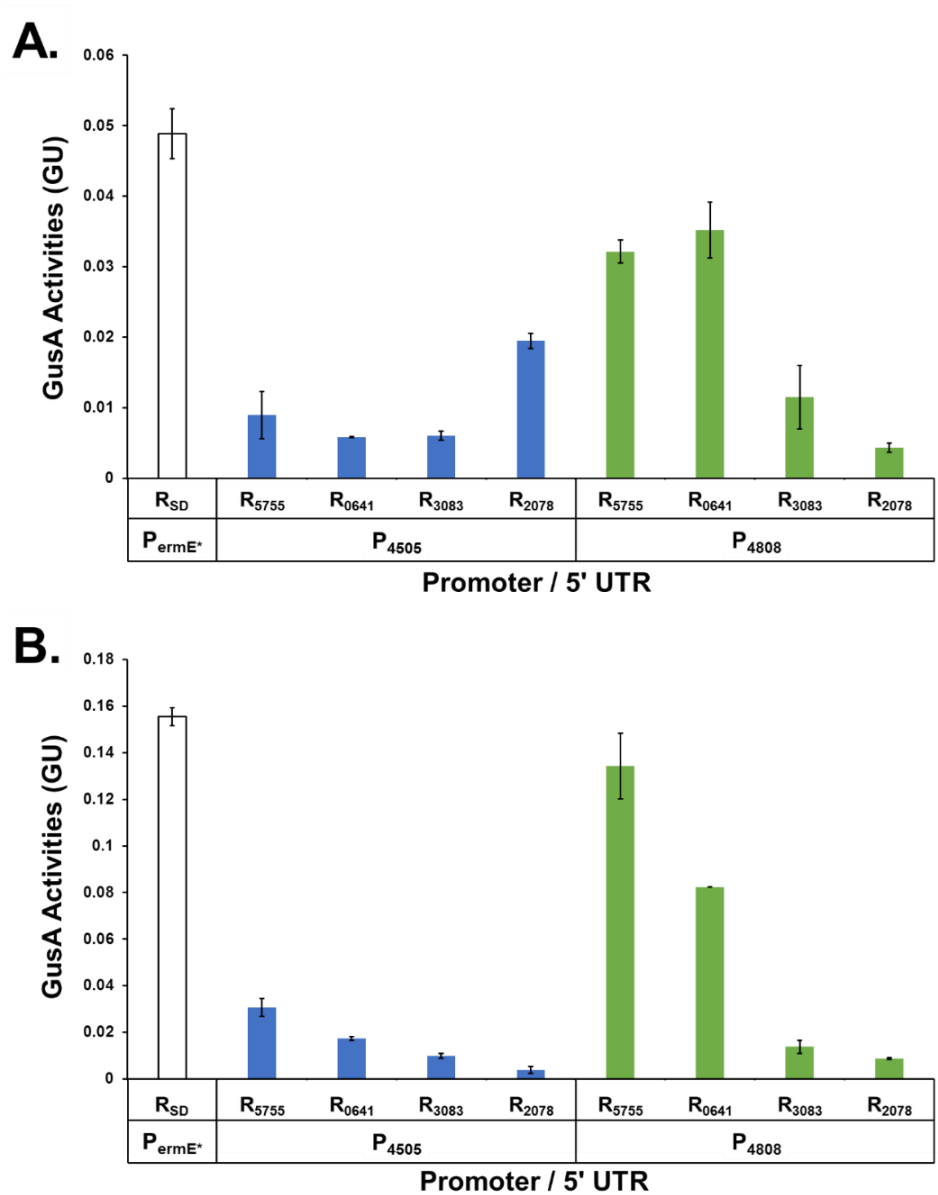


Figure 2.7. The eight promoter / 5' UTR combinations evaluated in (a) *Streptomyce venezuelae*, and (b) *Streptomyces avermitilis.* The same pIBR25 plasmids containing the eight combinations of promoter / 5' UTR and GusA were used. Promoter of SCO4505 was not as functional as expected in both *Streptomyces* strains.

The result could have been due to the differences in growth rates and gene regulations. As growth rates of the three *Streptomyces* species are different greatly from one another (Wang et al., 2013), the regulations of a cold shock protein, SCO4505, may be also different. The mechanisms of cold shock response are not well characterized in all the three species that it is hard to draw out definite explanations. Another possibility is that construction of expression systems using TSS information be very specific to organisms owing to strain specific nature of RNA-seq, Ribo-seq, and TSS-seq datasets.

2.4.3 Replacements of 5' UTR sequences from the previously reported promoters, $P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$

Not all promoters would be functional in different types of *Streptomyces* sp. or culture conditions as seen with P_{4505} that was only functional in *S. coelicolor*. To examine whether or not our method could be applied to solve such problems, three previously published promoters were engineered to demonstrate effectiveness of gene expression enhancement using our method, comparing the results to a method used to construct original $P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$.

$P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$ were known to be used in various *Streptomyces* sp. (Li et al., 2015), but based on our RNA-seq and Ribo-seq information, their usages were also limited to SMM, maltose-yeast extract-malt extract media (MYM), and tryptic soy broth (TSB). Average RPKM values of SCO5768, SCO4658, and SCO3410 promoters are all under 100 when grown in $R5^-$

complex media (Table 2.7). Compared to the gene expression profiles acquired from SMM, MYM, and TSB cultures, the strengths of the three SCO5768, SCO4658, and SCO3410 promoters were reduced dramatically. Moreover, $P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$ contain their own 5' UTR nucleotide sequences from -1 bp to -500 bp of each start codon. As a result, they are still under the influence of original 5' UTRs.

To enhance the gene expressions from the three promoters, each of the original 5' UTR sequences, from TSS to the 3' end of 5' UTRs, were replaced with R_{5755} following our method, using the TSS information. Since the three promoters are not silent in the cultures from $R5^-$ complex media, 5' UTR engineering was effective enough to enhance gene expression from $P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$. When the original 5' UTRs were replaced with R_{5755}, the expression levels of GusA from $P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$ were increased by 11.29, 8.61, and 3.97 folds, respectively (Figure 2.8) in $R5^-$ complex media. Strengths of the SCO5768, SCO4658, and SCO3410 promoters were so weak that their expression levels were not as strong as from that of the control, $P_{ermE}R_{SD}$. However, the three promoters were successfully engineered to show more effective gene expressions of interests under various culture conditions.

Table 2.7. RPKM and TE Values from Previously Reported Promoters

	RPKM				TE			
	EE[a]	ME[b]	LE[c]	S[d]	EE[a]	ME[b]	LE[c]	S[d]
SCO3410	78	80	39	27	0.29	0.20	0.46	0.35
SCO4658	105	103	46	31	0.36	0.38	0.19	0.32
SCO5768	27	48	78	142	0.47	2.06	2.56	1.46

a. EE: Early exponential phase
b. ME: Mid exponential phase
c. LE: Late exponential phase
d. S: Stationary phase

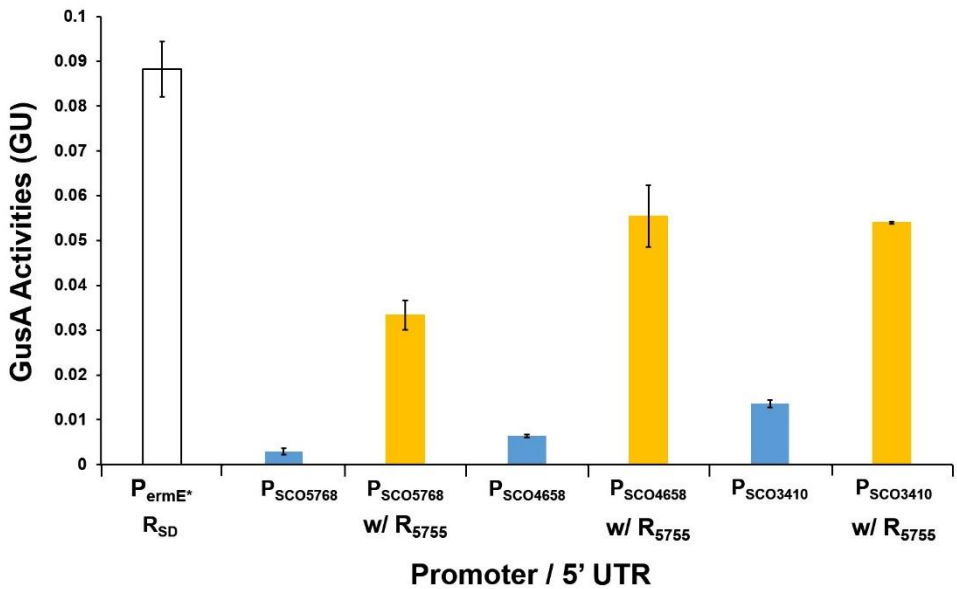


Figure 2.8. Evaluations of the original $P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$, and $P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$ containing 5' UTR of R_{5755}.

2.4.4 Applications of promoter and 5' UTR combinations for actinorhodin productions

To apply the promoter / 5' UTR combinations to overproduce a polyketide secondary metabolite as examples, ActII-orf4 and MetK were overexpressed with a strong, $P_{4505}R_{5755}$, and a weak, $P_{4808}R_{3083}$, combinations, respectively. ActII-orf4 is a well-known regulator (Wei and Newman, 2002) showing positive effects on the production of ACT. Therefore, overexpression of ActII-orf4 is expected to increase the production of ACT.

Overexpression of ActII-orf4 under its native promoter in a single plasmid was excluded from the experiments because this study was focused on constitutive promoters and 5' UTR with constitutive TE. According to RNA-seq data, it was also confirmed that promoter of ActII-orf4 is not constitutive. Transcription of ActII-orf4 usually started when the cell growth reached near stationary phase, suggesting that it is not constitutive, but only effective in stationary phase.

First, growths of actII-orf4 overexpressing mutants were slightly faster than that of control, WT containing blank pIBR25 plasmid, but there were no overall differences (Figure 2.9a). ACT production using $P_{4808}R_{3083}$ combination was about the same as that of the control. However, in the case of $P_{4505}R_{5755}$ combination, about 2.6 folds higher ACT production was observed, which corresponds to about 39% increase in ACT production compared to that of ermE*P overexpressed ActII-orf4 with its own 5'UTR region (Figure 2.9b).

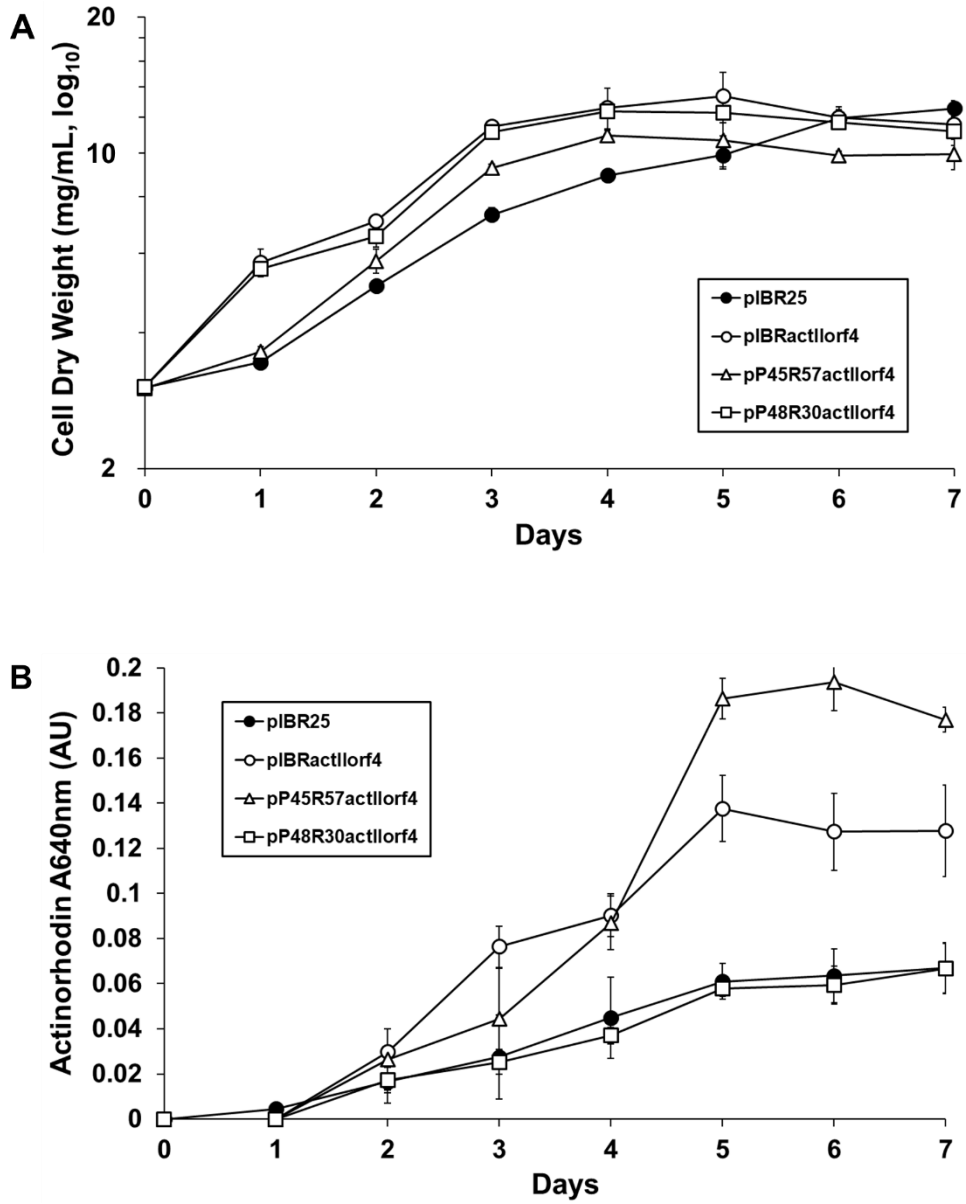


Figure 2.9. Growth and actinorhodin production curve of ActII-orf4 overexpression mutants. For all cases, *Streptomyces coelicolor* M145 with empty pIBR25 plasmid was used as a reference. (a) Growth and (b) actinorhodin productions.

For MetK, however, fine tuning of expression optimization was required for the maximum production of ACT. MetK is an enzyme that produces *S*-adenosylmethionine (SAM) from ATP and methionine, and is an essential gene for cell growth (Wei and Newman, 2002). Because SAM was known to activate various positive regulators of secondary metabolism in many different *Streptomyces* sp. (Zhao et al., 2010), MetK was often overexpressed. Appropriate SAM concentrations for polyketide antibiotics overproduction vary according to different *Streptomyces* strains in the ranges of 10 μM to 1 mM of external feeding (Huh et al., 2004). In the case of *S. coelicolor*, SAM was known to up-regulate ActII-orf4, and appropriate SAM concentration for the best up-regulation was about 2 μM of external treatment, which was very low compared to the other *Streptomyces* strains (Park et al., 2005).

When MetK was overexpressed with either a strong or weak expression system, the lag phase in growth of the weakly expressed MetK mutant using $P_{4808}R_{3083}$ combination was about a day longer than the other two strains, but the growth was recovered near the stationary phase (Figure 2.10a). ACT production was the highest with the weak $P_{4808}R_{3083}$ system, which is three folds increase compared to that of the control. In the case of the strong $P_{4505}R_{5755}$ overexpression system, ACT production was increased by 1.87 folds (Figure 2.10b). Overexpression of MetK is different from feeding SAM externally, but it is quite agreeable with the previous reports saying that weakly overexpressing MetK resulted in higher ACT productions.

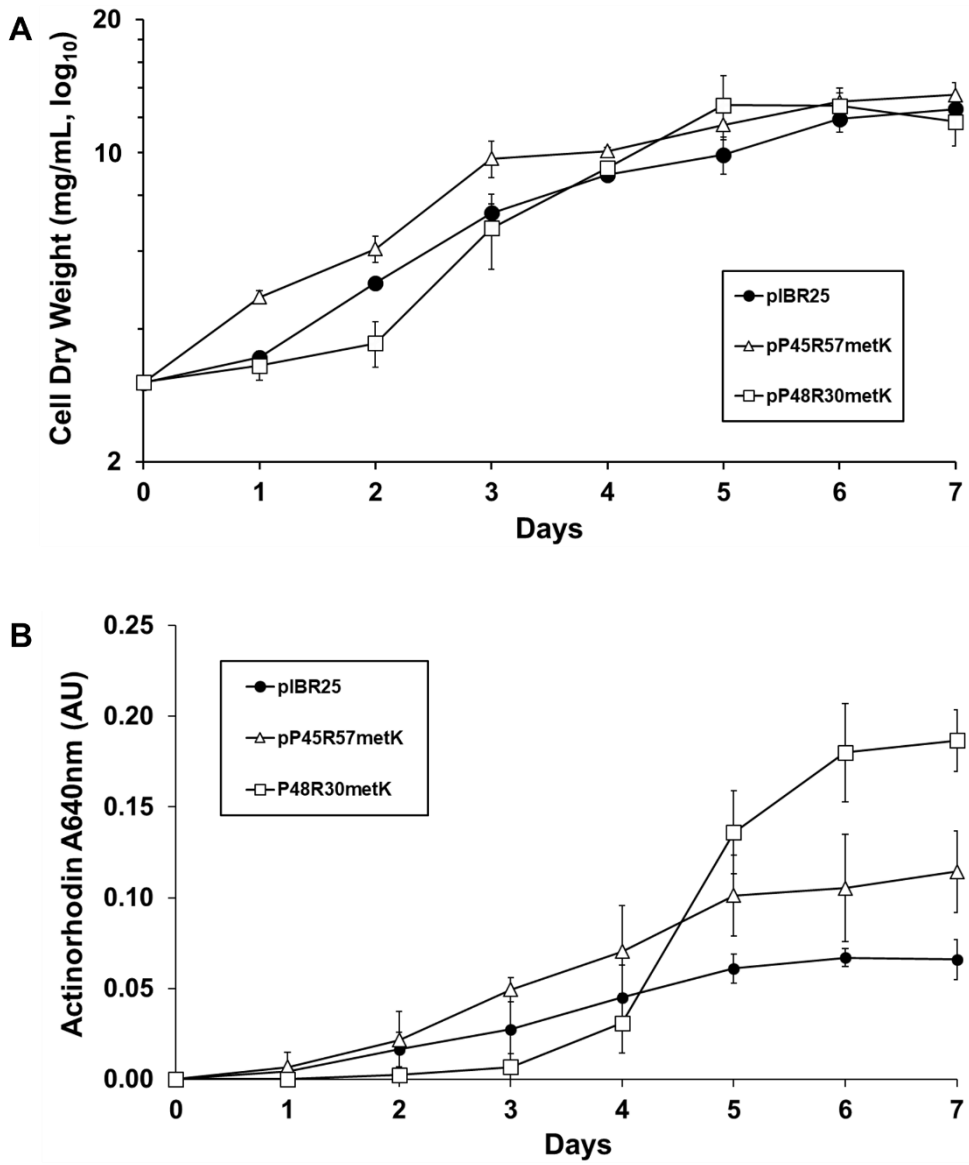


Figure 2.10. Growth and actinorhodin production curve of MetK overexpression mutants. (a) Growth and (b) actinorhodin productions.

Sporulation abilities of the two MetK overexpressing mutants were also compared because the effect of MetK on delaying sporulation is also known in *Streptomyces* strains (Ochi and Freese, 1982; Kim et al., 2003). When $R5^-$ complex agar media were used, the MetK mutant using $P_{4808}R_{3083}$ combination started to sporulate on day two just as in WT, whereas, $P_{4505}R_{5755}$-*metK* mutant began its sporulation around day three (Figure 2.11). The strongly expressed MetK mutant with $P_{4505}R_{5755}$ resulted in incomplete sporulation, similar to the result from previous reports (Kim et al., 2003).

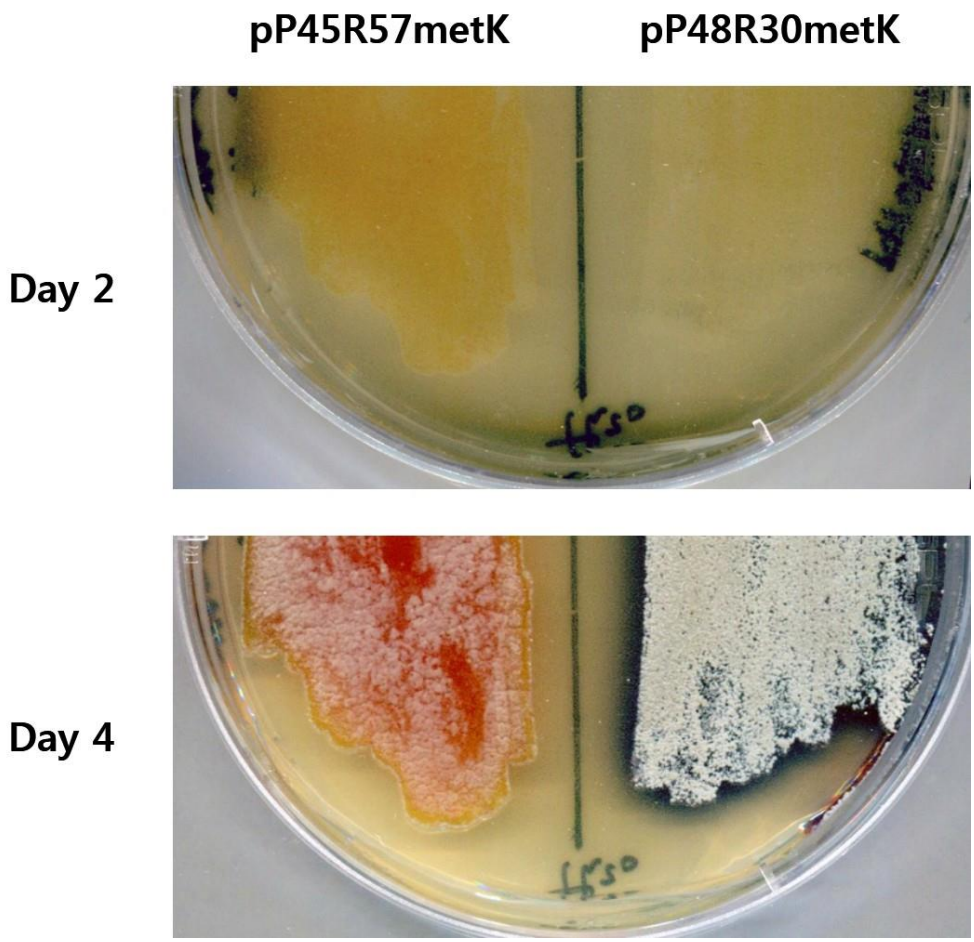


Figure 2.11. Growth of the two *metK* overexpressing *Streptomyces coelicolor* M145 strains on R5$^-$ complex agar media. A lag phase of weakly overexpressing *metK* strain was about a day longer than that of the others, just as in the liquid culture, but there was no alterations in abilities to sporulate.

2.5 Conclusion

In summary, promoters and 5' UTRs were screened, designed, and constructed based on three deep sequencing datasets. Often there are limitations in promoter evaluation and screening based only on RNA-seq data, because protein expression is also dependent on TE, and there are almost no well characterized RBS or 5' UTR sequences available, especially for *Streptomyce* strains. Total eight promoter / 5' UTR combinations were designed where candidate genes for promoter and 5' UTRs were selected based on RNA-seq, Ribo-seq, and TSS-seq. Using TSS information, the eight combinations were constructed, so that there were minimal interferences between promoters and 5' UTRs.

When the eight promoter / 5' UTR sets were evaluated with GusA, GusA was expressed in the ranges of 0.003 GU to 0.23 GU, similar to the order of the predictions made. GusA expressions from the eight combination sets covered ranges from about 0.03 folds to 2.4 folds of the reference, ermE*P / R_{SD} combination, which can represent most of the constitutive promoter / 5' UTR combinations available in this species. The eight combinations were also functional in two other media, YEME and SMM. These expression systems were successfully applied to differentially express ActII-orf4 and MetK as demonstrations of its utilities. It is unfortunate for P_{4808} only working in *S. coelicolor*. But as shown with $P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$, a working promoter from literature or RNA-seq data for species of interests could be found in place of P_{4808}.

It is expected that the expression system developed herein could reduce the gap between genetic design with metabolic modeling (Kim et al., 2014; Kim et al.,

2016) and experimental implementation, providing a useful tool in metabolic engineering. This study extended and enhanced previous promoter searching methods from RNA-seq, and designed promoters and 5' UTRs that have minimal interferences between transcription and translations. As long as there are available RNA-seq, Ribo-seq, and TSS-seq data, our method can be applied in many different species. Unfortunately, one of the two promoters from *S. coelicolor* was not fully functional in *S. venezuelae* and *S. avermitilis*, suggesting that our method is still somewhat strain-specific. However, as shown with replacing 5' UTRs of $P_{SCO5768}$, $P_{SCO4658}$, and $P_{SCO3410}$, improvements in the system would be expected depending on the types of promoter used along with 5' UTRs, as the trends of translations from the four 5' UTRs were closely maintained within the three species. It would provide an insight to develop general methodology for constructions of various protein expression systems in other bacterial species with simple gene cloning manipulations.

Chapter 3.

Engineering *Streptomyces coelicolor* for Productions of Branched Chain Fatty Acids

3.1 Abstract

Branched chain fatty acids (BCFA), which have better properties as lubricants due to lower melting points compared to that of straight chain, were appealing target fatty acids (FA) for productions in microorganisms. Actinobacteria, especially *Streptomyces* species, have unique secondary metabolisms that they already produced BCFAs in high percentages in their membrane lipids unlike other bacteria. To increase productions of the BCFAs in *Streptomyces coelicolor*, competing pathways of fatty acids synthase (FAS) genes were eliminated by deletion of secondary metabolite synthesis gene clusters, and β-oxidation to block degradations of FA. 3-ketoacyl acyl carrier protein synthase III (KASIII) and branched chain α-keto acid dehydrogenase (BCDH) were overexpressed to accelerate initiations of FA synthesis, and supplementations of BCFA precursors. Leucine and vanillin were each supplemented in culture media to further increase FA productions, resulting in the highest production of 250 ± 43.5 mg/L. It was about 5 folds increases compared to that of the wild type (WT) *S. coelicolor*.

Keywords: *Streptomyces coelicolor*, Branched-chain fatty acids, KASIII, BCDH

3.2 Introduction

Productions of renewable fuels and sustainable energies are in great demands because there are limited amounts of resources due to growing populations and industries (Xu et al., 2017). Biofuels from biomasses and wastes of microorganisms (i.e. *Escherichia coli* and yeasts) have been appealing targets of engineering (Koppolu and Vasigala, 2016). Fatty Acid (FA) is one of the most studied targets because it can be modified into various types of biofuels (i.e. alcohols and alkane gas) (Zhou et al., 2014). Productions of branched chain fatty acids (BCFAs) began to attract attention because of their advantageous chemical properties over straight chain fatty acids (SCFAs). BCFAs are preferred biofuels for uses in cold environments because they have lower freezing points than SCFAs do (Bentley et al., 2016). Properties of BCFAs make themselves good lubricants as well (Bart et al., 2012). Due to that reason, recent studies began to engineer microbes as host strains for productions of BCFAs. Among several engineering targets, branched chain amino acids (BCAAs) catabolism was the major component for the productions of BCFAs.

BCAA catabolites, isobutyryl-CoA, isovaleryl-CoA, and 2-methylbutyryl-CoA, which are from valine, leucine, and isoleucine respectively, become starter units for BCFAs synthesis (Kaneda, 1991). Isobutyryl-CoA becomes iso-form even-chain BCFAs (i.e. isopalmitate), isovaleryl-CoA becomes iso-form odd-chain BCFAs (i.e. isopentadecanoate), and 2-methylbutyryl-CoA becomes anteiso-form odd-chain BCFAs (i.e. anteisopentadecanoate) (Body, 1984) (Figure 3.1).

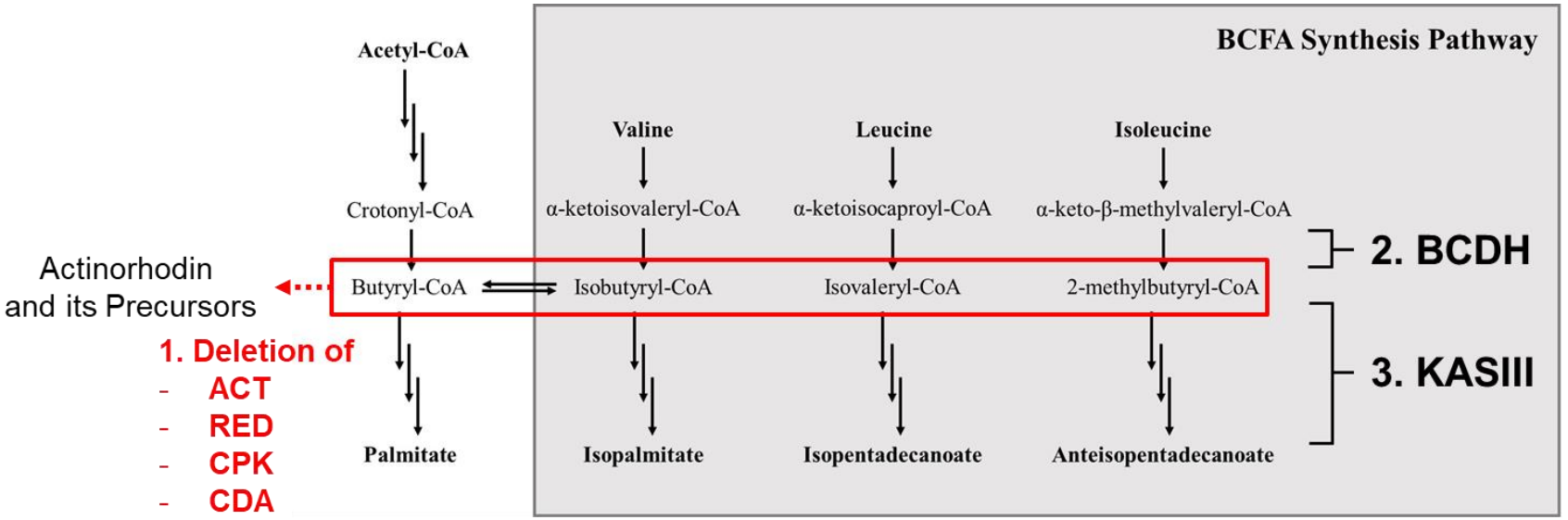


Figure 3.1. BCFA synthesis pathway from BCAA and acetyl-CoA. Types of FAs produced are specific to the amino acids metabolized by BCDH.

3-ketoacyl acyl carrier protein synthase III (KASIII) of FAS genes binds the three branched chain acyl-CoAs as starter units, and from there, chain elongations occur using malonyl-CoA as an elongation unit (Revill et al., 1996). In other words, SCFAs are mainly synthesized without BCAA catabolism. As a result, engineering of a BCAA degradation pathway would be a key to increase productions of BCFAs. In recent studies, BCAA catabolism pathways was first introduced to *Escherichia coli* in order to produce BCFAs.

E. coli is one of the most widely used host for FA productions for easiness in gene engineering, but it cannot produced BCFA despite the fact that *E. coli* can produce gram scale amounts of SCFAs and hydroxyl fatty acids (Sung et al., 2015). *E. coli* lacks genes, which can utilize BCAA catabolites towards BCFA productions. *E. coli* KASIII does not have any substrate specificities towards branched chain acyl-CoAs (Choi et al., 2000), but it only accepts acetyl-CoA. As a result, KASIII and BCDH from other microorganisms (i.e. *Bacillus subtilis* and *Staphylococcus aureus*) must be heterologously expressed in *E. coli* for the productions of BCFAs. In order to produce about 180 mg/L of BCFAs from glucose, 15 enzymes were overexpressed in *E. coli* (Bentley et al., 2016). For that reasons, we were interested in microorganisms that could utilize BCFAs better than *E. coli* could, and *Streptomyces* species attracted our attention.

Characteristics of *Streptomyces* sp. provide easiness in gene engineering, and exhibit great potentials as a host strain for BCFA productions. Acyl-CoA building blocks come from both glucose and BCAA catabolism, and their pathways are better developed and characterized than that of *E. coli* (Howard et al., 2013). In

Streptomyces coelicolor, about 70 ~ 75 % of entire fatty acid pools are composed of branched chain fatty acids. The most common fatty acids in *S. coelicolor* is isopalmitate, accounting for about 20% of total FAs (Li et al., 2005). There are no needs for replacements and optimizations of KASIII, and reconstructions of BCAAs catabolism pathways unlike in *E. coli*. For overproductions of BCFAs, reductions of major competing pathways are also possible.

Streptomyces sp. are well known for their abilities to produce numerous secondary metabolites such as antibiotics (McKenzie et al., 2010). Of all, polyketide secondary metabolites are one of the most well-known compounds produced by *Streptomyces* sp.. Polyketides are produced by polyketide synthase (PKS) genes using the same elongation units as fatty acid synthase (FAS) genes, acyl-CoAs (Gajewski et al., 2017). As a result, increasing acyl-CoA fluxes into FAS, while reducing the fluxes towards PKS, may result in overproductions of desired BCFAs (Tanaka et al., 2017). In this study, we developed *S. coelicolor* as a host strain for BCFA productions by utilizing BCAA catabolism. Preliminarily, BCAAs supplementation, KASIII and BCDH overexpression, and Δ*fadD* deletion were tested in Δ*actII-orf4* deletion mutant to observe their effects on BCFAs synthesis. And then, the same strategies were applied to *S. coelicolor* M1146 to maximize the BCFAs production.

3.3 Materials and Methods

3.3.1 Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used throughout the study are described in Table 3.1. For cultures of *E. coli* strains, Luria-Bertani (LB) Broth (Becton Dickinson, USA) was used along with appropriate antibiotics when necessary. $R5^-$ complex medium made by standard protocols (Kieser et al., 2000) were used for cultures of *Streptomyces* strains. Difco Nutrient Agar (DNA) medium (Becton Dickinson, USA) and Mannitol Soya flour (MS) agar media, containing 20 g/L mannitol, 20 g/L soya flour, 20 g/L agar in 1 L of distilled water, were used for conjugations of cosmids into *Streptomyces* strains from *E. coli* ET12567. Appropriate antibiotics were also used for selections of mutants. Antibiotics were used in concentrations of 0.1 mg/mL ampicillin, 8 μg/mL thiostrepton, and 50 μg/mL apramycin. For removals of *E. coli* from conjugated *S. coelicolor*, 25 μg/mL nalidixic acid was used for cultures on agar plates.

Table 3.1. List of bacterial strains and plasmids used in this study

	Descriptions	References
Streptomyces strains		
Streptomyces coelicolor A3 (2) M145	WT strain	Kim et al., 2014
Streptomyces coelicolor M1146	ΔactΔredΔcpkΔcda strain	Coze et al., 2013
E. coli strains		
DH5α	a host for gene constructions	ThermoFisher
JM110	a strain for demethylation of plasmids	Stratagene
ET12567	a strain for conjugations of *S. coelicolor* cosmids	ATCC
Plamids		
pSupCos5085	*actII*-orf4 deletetion cassette cloned into *Eco*RI/*Xba*I site of pSupercos1	this study
pIBR25	*Streptomyces* vector with SCP2* origin; Amp^R and *tsr* marker	Yi et al., 2017
pIBR25::ME	ME cloned into *Eco*RI/*Hind*III sites of pIBR25	Kim et al., 2016
pIBR25::BCDH	BCDH cloned into *Xba*I/*Eco*RI sites of pIBR25	Kim et al., 2014
pIBR25::KASIII	KASIIIcloned into *Pst*I/*Xba*I sites of pIBR25	this study
pIBR25::KASIII::BCDH	pIBR25 containing KASIII (*Pst*I/*Xba*I) and BCDH (*Xba*I/*Eco*RI)	this study
pIJ773	A template vector for amplifications of gene deletion cassettes	Yang, et al., 2009
pUWLCRE	*Streptomyces* plasmid containing *cre* recombinase gene	Swiatek et al., 2013
pdCos6196	*fadD* deletion cassette inserted and reaplced *fadD* of St2G5.17 cosmid	this study
pP48R30	pIBR25 containing $P_{SCO4808}R_{SCO3083}$ in *Sac*I/*Xba*I sites instead of PermE*	Yi et al., 2017
pP48R30::BCDH	BCDH cloned into *Eco*RI/*Hind*III sites of pP48R30	this study
pP48R30::KASIII	KASIIIcloned into *Eco*RI/*Hind*III sites of pP48R30	this study
pP48R30::KASIII::BCDH	KASIII::BCDH cloned into *Eco*RI/*Hind*III sites of pP48R30	this study
pIBR25::NdgR	NdgR cloned into *Bam*HI/*Hind*III sites of pIBR25	Yang, et al., 2009
pIBR25::ilvBCD	*ilvBCD* cloned into *Xba*I/*Hind*III sites of pIBR25	this study
Cosmids		
St2G5.17	a *S. coelicolor* cosmid containing *fadD*	this study

E. coli and *Streptomyces* strains were cultured with 200 rpm shaking in Lab Companion SK-71 Benchtop shaker (Jeio Tech, South Korea), each in 37 ℃ and 30 ℃ respectively. For *Streptomyces*, 200 mg of wet weighted cells from overnight 50 mL $R5^-$ seed cultures using 250 mL flasks were inoculated to fresh 50 mL $R5^-$ media for productions of both ACT and BCFAs. Final 2 mM of vanillin, valine, leucine, isoleucine, and vanillin were added at this point, when needed. 5 mL of cell culture were harvested and dried in a 60 ℃ oven for calculations of dry cell weights (DCW).

3.3.2 Strain construction

A deletion cosmid for *actII-orf4* was constructed using pSupercos1 vector. Primers 1 and 2 were used to PCR amplify upstream region of *actII-orf4*. Primers 3 and 4 were used to clone LoxP and apramycin resistance gene. Primers 5 and 6 were used for downstream region of *actII-orf4*.

Malic enzyme (ME) was PCR amplified and cloned into *Eco*RI and *Hind*III sites of pIBR25::BkdABC (Kim et al., 2014) using primers 7 and 8. PCR Amplified KASIII with primers 9 and 10 was inserted into *Pst*I and *Xba*I sites of pIBR25, resulting in pIBR25::FabH. PCR amplified *bkdABC*, primers 11 and 12, was inserted into pIBR25::FabH to created KASIII and BCDH double overexpression plasmid. Deletion cassettes for *fadD* were constructed using primers 13 and 14, and pIJ773 plasmid as a template for antibiotics marker. Sequence information about all the primers used in this study are listed in Table 3.2.

Table 3.2. List of primers for strain constructions

	Sequence
1	ATATGAATTCTGCGCACCGTCGTCAACGT
2	ATATTCTAGACATCTGCGCCCCCGTCGA
3	ATATGAATTCTATATACTAGTGTCGACCCATAACTTCGTATA
4	ATATATTCTAGACTGCTTCGATAACTTCGTATA
5	ATATGAATTCTATATACTAGTAAGGTGCTCGTGTAGCACC
6	ATATTCTAGAGAGCCGTGCGCGTTGATGTA
7	ATTATGAATTCCCCTCAAACCATGAACTCTTA
8	ATAATAAGCTTTCACCGGCGCGCAACCCCCTC
9	ATTATCTGCAGGAAGGATGCGGTTACACGGTCCGAGGAGGGA GCCACCGATGTCGAAGATCAAGCCCAGCAAG
10	ATTATTCTAGATGATCCGGCACGGAGTGCCTAG
11	ATAATCTAGACCCACCCTTGTGCGTCCG
12	ATTATGAATTCTCAGGCCCAGCTGATCAACCGGCT
13	CGGTTCCGCGAACGAGTACCGCGAGGAGGACTTCCGGTGATT CCGGGGATCCGTCGACC
14	TCAAGCTGAGTCCGAAACAGTCGGTGGCCTTTTCCGTCATGTA GGCTGGAGCTGCTTC
15	ATATAGAGCTCCGGCACCGGCACCTCGTGGC
16	ATATA GAATTC ATGTCGAAGATCAAGCCCAGCAAG
17	ATATATCTAGAATGTCGAAGATCAAGCCCAGCAAG
18	ATTAT AAGCTT TGATCCGGCACGGAGTGCCTAG
19	ATATA TCTAGA GTGACCGAGAGCACTGCCGCGC
20	ATTAT AAGCTT TCAGGCCCAGCTGATCAACCGCT

To replace ermE* promoter with weaker expression system, $P_{SCO4808}R_{SCO3083}$ (Yi et al., 2017) was PCR amplified with primers 15 and 16, and was inserted into *Sac*I and *Xba*I site of pIBR25, resulting pP48R30. *fabH* and *bkdABC* were PCR amplified using primers 17 and 18, and 19 and 20 respectively, and *fabH*::*bkdABC* was PCR amplified with primers 17 and 20 using pIBR25::FabH::BkdABC as a template. The three amplified fragments were inserted into *Eco*RI and *Hind*III sites of pP48R30.

Deletion of *actII-orf4* was performed by the standard protocol (Kieser et al., 2000). After apramycin resistance marker was removed from *actII-orf4* deletion mutant using *cre* recombinase gene, deletion of *fadD* gene was performed following the same standard protocol. All other overexpression plasmids were transformed into *S. coelicolor* by protoplast transformation methods (Kieser et al., 2000).

3.3.3 Fatty acids extractions and quantifications

From 50 mL of shake flask cultures, 5 mL samplings were made on day 3 and 6. 200 μL acetic acid was added to each samples, and then, 10 mL of 1:1 chloroform to methanol solution was added. Aqueous layer was removed from the samples, and the left over organic layer was completely dried. The fatty acid extracts were then reconstituted with 1mL of 5% H_2SO_4 in methanol. 6.75 μL of 10 mM C17 were added to the samples at this point as an internal standard. Methyl esterification was performed by heating up the samples for 2 hours at 90 ℃ in sealed vials. Fatty acid methyl esters (FAME) were then extracted with 200 μL hexane after 1 mL of 0.9% NaCl in water was added to the methyl esterified samples. All the authentic fatty

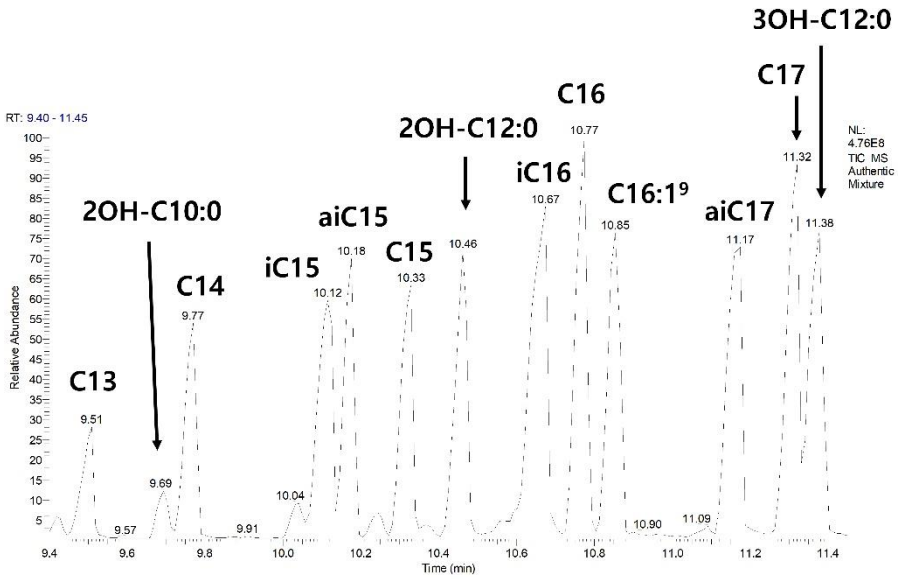
acids for drawing out quantification curves were purchased from Sigma-Aldrich, USA (Bentley, et al., 2016).

FAME were analyzed and quantified by GC-MS, a Trace GC Ultra Gas Chromatograph (Thermo scientific, USA) connected to an ion trap mass detector, ITQ1100. TR-5MS column with 30 m X 0.25 mm ID X 0.25 μm film (Thermo scientific, USA) was used for separations of FAME. GC oven started its temperature from 50 ℃, which was held for 1 min. The temperature was increased at a rate of 20 ℃/min up to 250 ℃. It was held at 250 ℃ for 10 minute before a new cycle began. It successfully separated FAMEs (Figure 3.2). Fatty acid profiles were compared to that of authentic samples for peak identification and quantification.

3.3.4 RNA-seq data

RNA-seq data used to select a deletion target for removal of β-oxidation pathway was from the previously published study (Jeong et al., 2016). We used the raw data deposited in the Gene Expression Omnibus archive with accession code GSE69350.

A



B

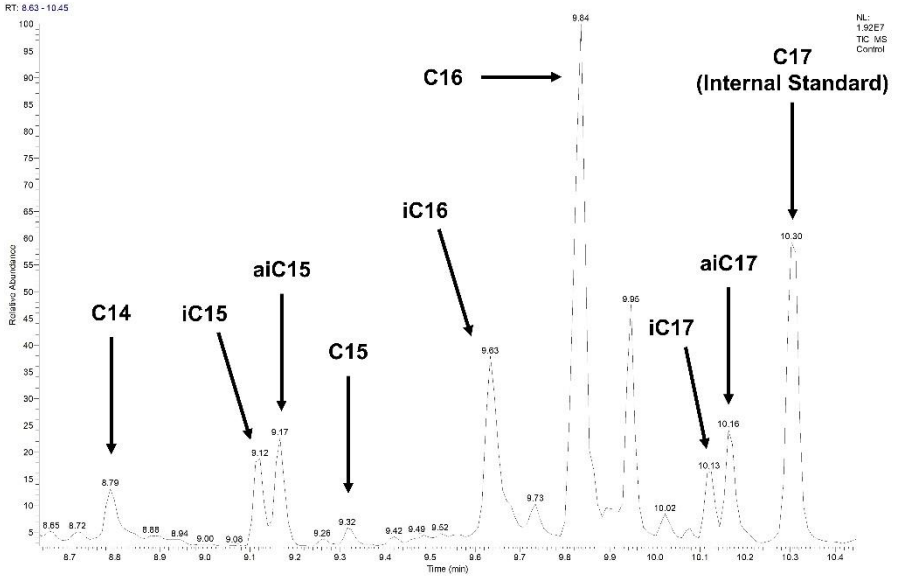


Figure 3.2. GC-MS chromatogram of (a) mixtures of authentic samples, and (b) FA extracts from *S. coelicolor*.

3.4 Results and Discussion

3.4.1 BCAA supplementation for fatty acid productions in *actII-orf4* deletion mutant

ACT is the most produced polyketide antibiotics in *S. coelicolor* (Kim et al., 2014), and its production is known to be regulated by several factors (i.e. S-adenosylmethionine and bld sigma factors) (Okamoto et al., 2003). Among them, deletion of *actII-orf4*, ACT pathway specific positive transcriptional regulator (Arias et al., 1999), was previously reported that it vanished transcriptions of ACT PKS genes without effecting any cell morphology (Zhang et al., 2009). Since the absence of *actII-orf4* does not completely vanish ACT producitons, it was a good deletion target for studies of relationships between FA and polyketide biosynthesis in *S. coelicolor*.

First, to confirm whether Δ*actII-orf4* (SCO_{dA}) and BCAAs have effects on BCFA productions, FA productions with BCAA supplementations were measured. First, there were no differences in growths of the control, WT with a blank pIBR25, and SCO_{dA}. Growths of SCO_{dA} with BCAA supplementations were almost identical, except for leucine (Figure 3.3a). DCW of SCO_{dA} with leucine were doubled on day 2 of the culture (i.e. DCW = 6.37 ± 0.35 g/L), and reached to stationary phase one to two days faster. The deletion of *actII-orf4* increased the total FA production by 1.5 folds (Figure 3.3b) and 1.3 fold (Figure 3.3c) on the day 3 and 6 of cultures respectively.

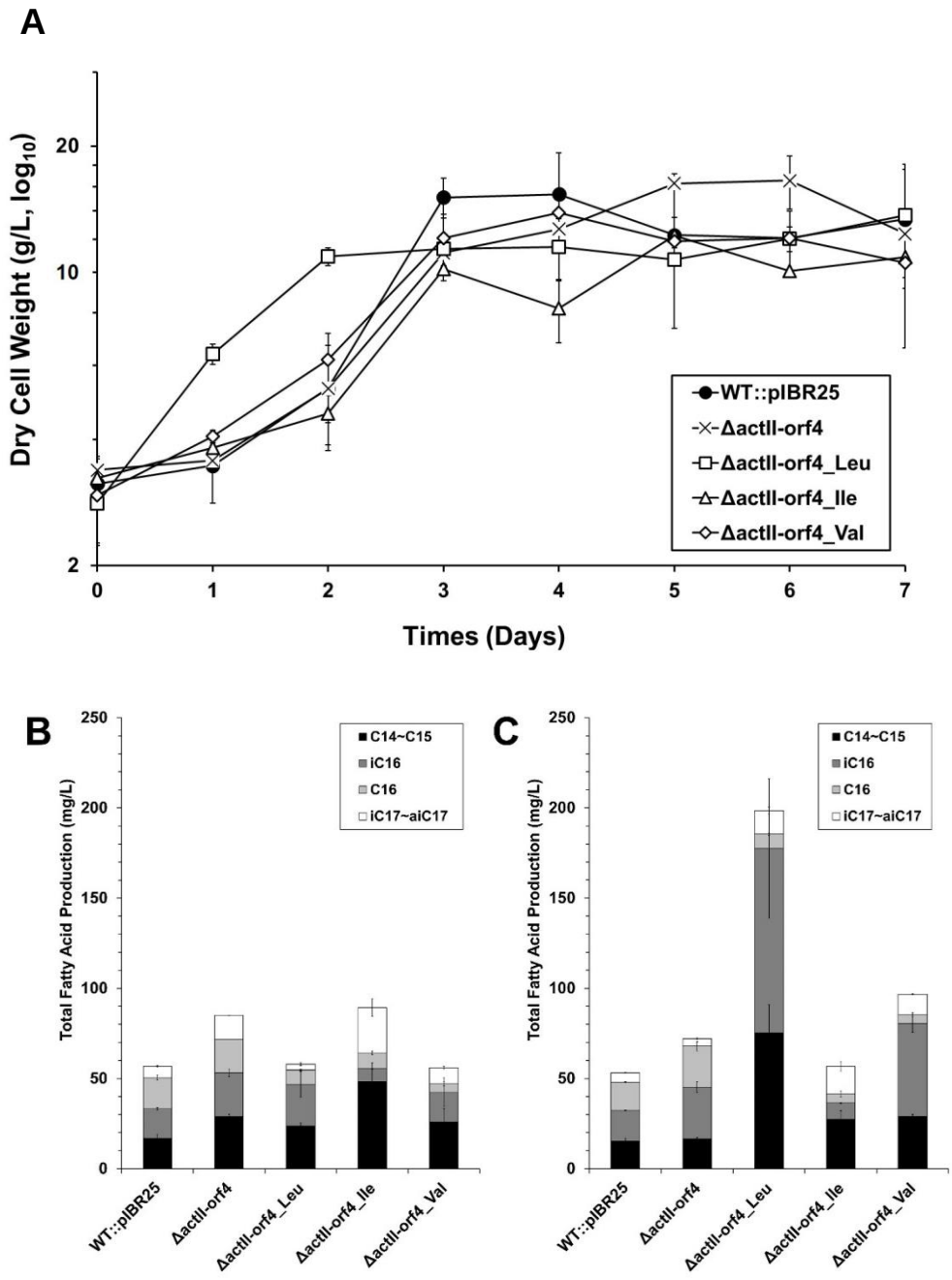


Figure 3.3. (a) Growths curves, and BCFA productions on (b) day3 and (c) day 6 of Δ*actII-orf4* cultures with BCAA supplementations. WT with a blank plasmid and Δ*actII-orf4* without BCAA supplementation were used as a control.

BCAA supplementation did not result in a huge increases of BCFA productions on day 3. However, in all cases, contents of BCFA percentage increased from 56.9% WT to over 70%. On day 6 of the cultures, leucine supplementation in SCO_{dA}, increased the production of FAs up to 198 mg/L, with 93% BCFA, and valine supplementation up to 96 mg/L, 91.9% BCFA, which are 3.74 folds and 1.82 folds increases compared to that of the WT control respectively.

3.4.2 Fatty acid productions from Δ*actII-orf4* mutants

Major differences between *E. coli* FAS and *S. coelicolor* FAS comes from KASIII, the first enzyme in fatty acids biosynthesis pathway. *E. coli* KASIII only accepts straight chain acyl-CoAs, acetyl-CoA and propionyl-CoA (Choi et al., 2000). For that reason, *E. coli* cannot produce BCFAs. As a result, alterations in productions of fatty acids and their profiles from SCFA to BCFA or vice versa required cloning of *B. subtilis* KASIII into *E. coli* (Jiang et al., 2015; Bentley et al., 2016), or *E. coli* KASIII to *S. coelicolor* (Li et al., 2005). Since *Streptomyces* sp. already have abilities to produced BCFAs, there was no need for optimizing KASIII gene in *S. coelicolor*. Having such advantages, we overexpressed total three genes, including KASIII (SCO_{dAK}) and two genes that are previously known to increase ACT productions, ME (SCO_{dAM}) (Kim et al., 2016) and BCDH (SCO_{dAB}) (Kim et al., 2014) in SCO_{dA}. Their fatty acid productions were measured with GC-MS.

Overall cell mass of SCO_{dA}, SCO_{dAK}, SCO_{dAM}, and SCO_{dAB} were about the same as that of WT. Considering error ranges, their dry cell weights were about 10 g/L (Figure 3.4a). Interestingly, growth rates of the four mutants were different in

exponential phase, but their maximum weight were about the same at stationary phase. BCDH overexpression in SCO_{dA} did not result in increases of cell mass, unlike it did with that in WT. ACT productions in the four mutants decreased dramatically. SCO_{dA}, SCO_{dAK}, and SCO_{dAM} produced only a half of ACT that WT could produce. For SCO_{dAB}, ACT production decreased down by 10 folds from SCO_{B} (Figure 3.4b).

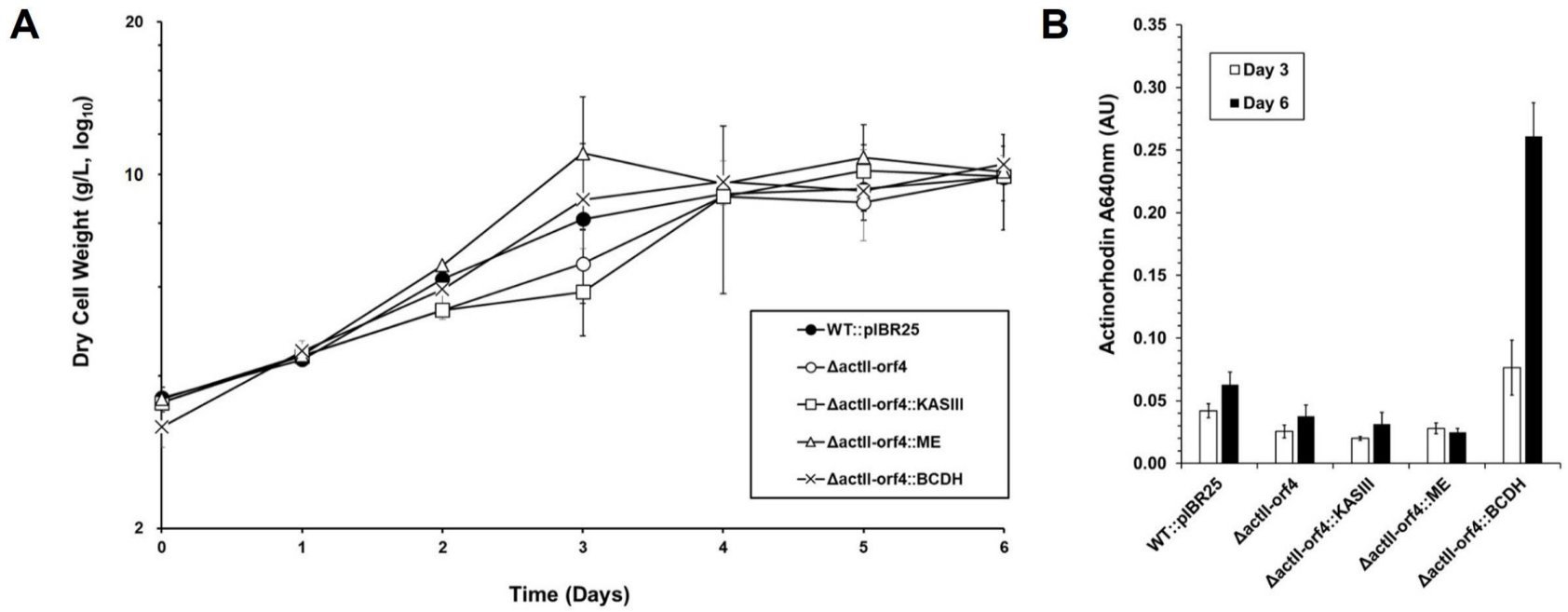


Figure 3.4. Growths and ACT productions from SCO_{dA} mutants. (a) Growth curves, (b) ACT productions of SCO_{dA} mutants.

Quantifications of BCFA were performed at two different time points, one at exponential phase, day 3, and the other at stationary phase, day 6. Overall fatty acid productions on the day 3 of the control was about 56.67 ± 5.13 mg/L. *actII-orf4* deletion increased the production to 85.05 ± 1.62 mg/L. The maximum total fatty acid production was further increased with SCO_{dAK}, resulting in 87.95 ± 12.11 mg/L, about 1.6 folds of WT (figure 3.5a and b). BCFA contents of the control was about 57.0% of total fatty acids. The percentage increased the most, about 78.6%, in SCO_{dAK}. Interestingly, KASIII overexpression mainly contributed towards changing ratio between BCFA and SCFA. KASIII overexpression increased BCFA contents from 71.7% in SCO_{dA} to 78.6%, which is about 37% increase compared to that of the control. When looked into specific lengths of fatty acids, isopalmitatae (iC16) resulted such increases in the total fatty acids productions.

Unfortunately, effects of ME or BCDH overexpression was not as dramatic as in ACT productions. Unlike BCDH overexpression increased ACT productions by 30 folds, it only increased fatty acids productions by 2 folds. The maximum FA productions were with SCO_{dAB}, 112.34 ± 4.79 mg/L, with 80% of BCFA contents, and again, iC16 was the most abundant FA.

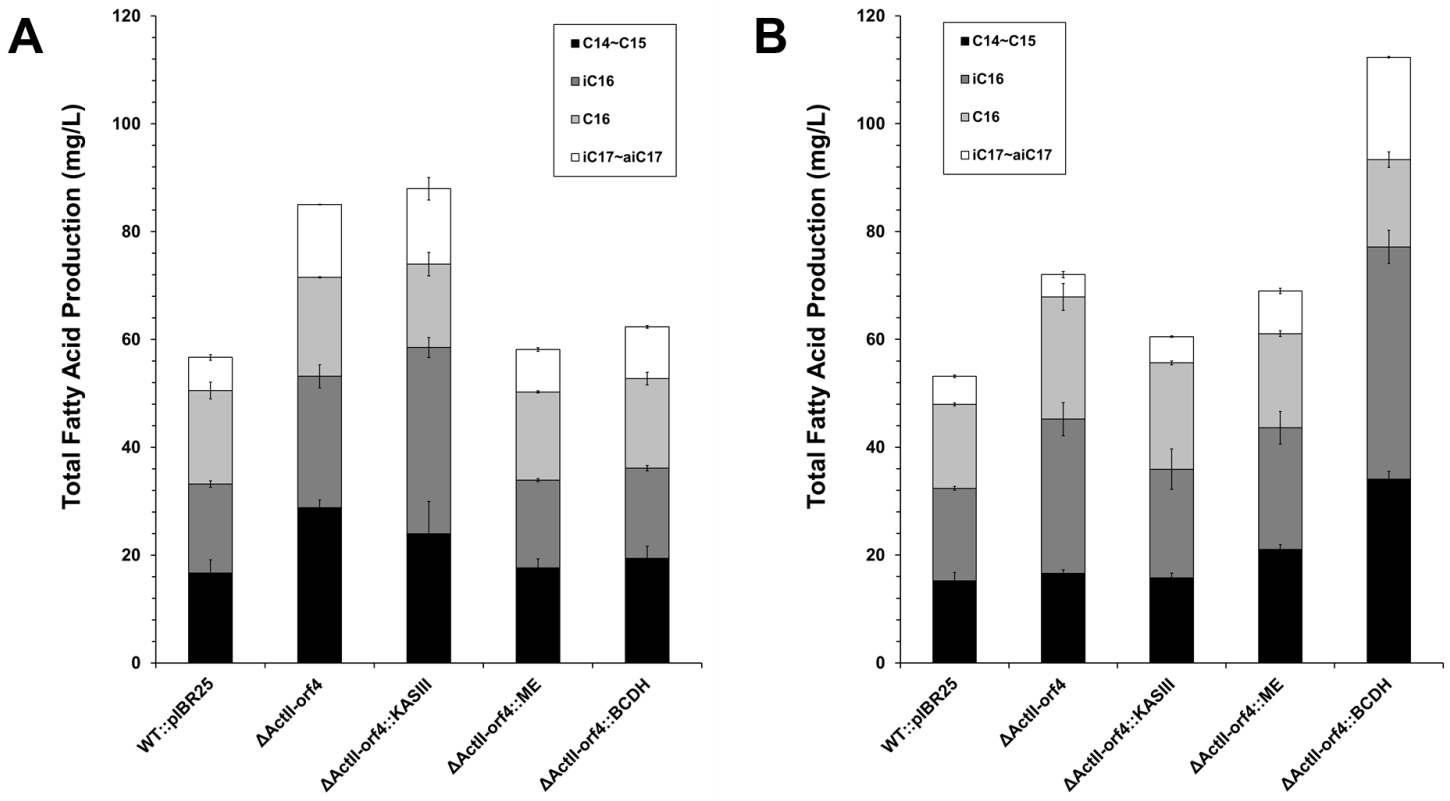


Figure 3.5. BCFA productions from SCO_{dA} mutants. BCFA productions on (a) day 3 and (b) day 6 of bacterial cultures.

Productions of isopalmitate was the most effected fatty acids throughout the study due to substrate specificities of KASIII. *Streptomyces* sp. can produce both SCFAs and BCFAs because their KASIII have broader substrate specificities (Magnuson, et al., 1993). *S. coelicolor* KASIII has the highest affinities towards isobutyryl-CoA with *k*cat/*k*m of 9.84 $\mu M^{-1}min^{-1}$, resulting in major productions of iC16 (Singh et al., 2012). FA profiles from SCO_{dA} mutants agreed with previous reports.

Towards the end of batch cultures, FA productions began to decrease, possibly explained by a presence of β-oxidation pathway. Actual gene transcription levels of β-oxidation related genes, acquired from RNA-seq of WT *S. coelicolor* grown in $R5^-$ complex media (Yi et al., 2017), increases towards stationary phase (Table 3.3). It means that β-oxidation pathway is activated towards the stationary phase of growths, and may explain the decreases in FA productions. β-oxidation supplies acetyl-CoA as a carbon source for growth from catabolism of FAs when C source in media become limited (Schulz, 1991). For that reason, β-oxidation is often deleted in *E. coli* FA production hosts (Lennen and Pfleger, 2012). As a result, we determined to remove β-oxidation pathway by deletion of *fadD*.

Table 3.3. List of β-oxidation pathway genes and their transcription levels

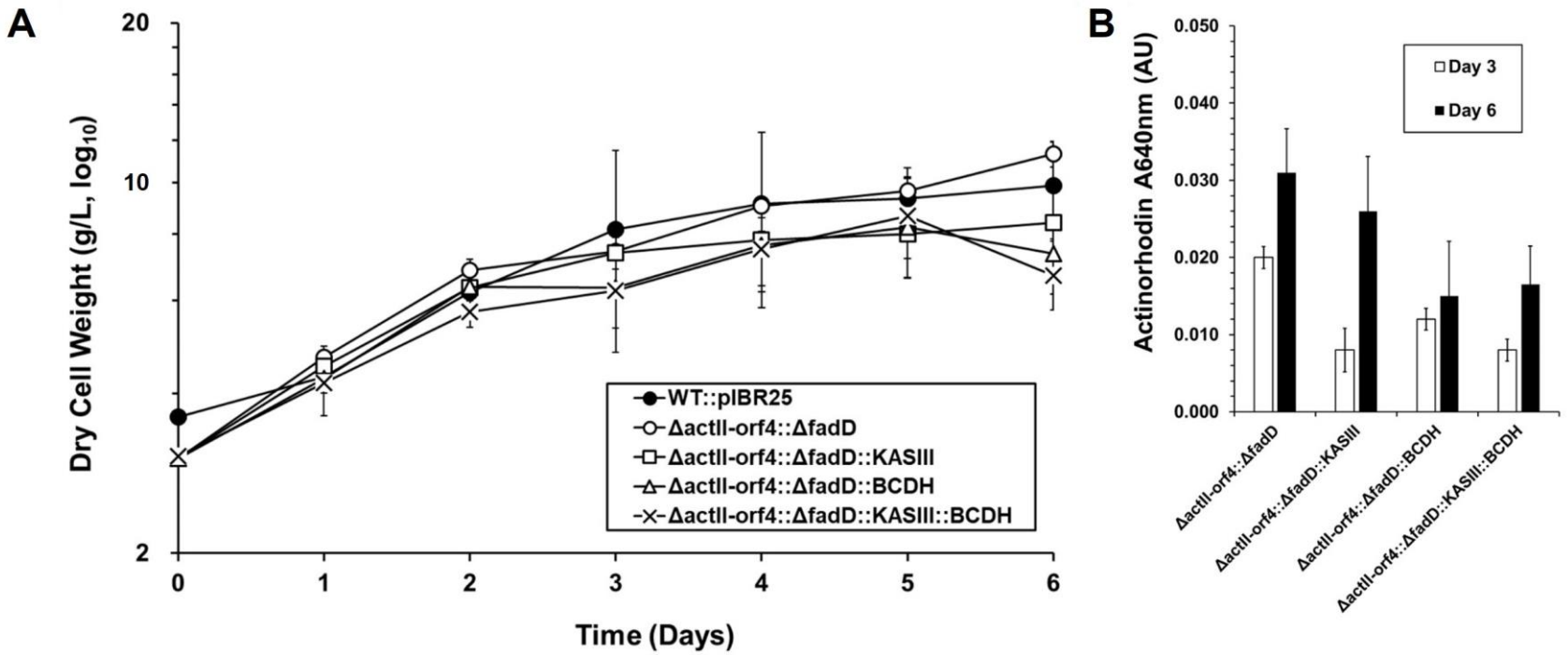
	Gene	EE[a]	ME[b]	LE[c]	S[d]	Average RPKM
SCO2131	FadD	53.71	40.93	22.68	26.03	35.84
SCO3051	FadE	83.71	154.17	339.12	334.08	227.77
SCO3079	FadA	24.86	30.66	44.14	77.67	44.33
SCO4006	FadK	11.04	23.78	35.89	47.25	29.489
SCO4384	FadB	5.28	4.58	4.77	7.14	5.44
SCO4502	FadA	4.38	4.57	2.08	2.25	3.32
SCO6026	FadB	178.92	125.67	129.88	154.73	147.30
SCO6196	FadD	11.06	73.81	35.39	189.07	77.33
SCO6732	FadB	13.48	21.50	31.47	33.86	25.07
SCO6789	FadB	1.71	1.37	1.44	1.98	1.62
SCO6968	FadD	3.66	5.19	4.07	2.97	3.97
SCO7329	FadD	2.10	5.20	27.76	46.45	20.38

a. EE: Early exponential phase
b. ME: Mid exponential phase
c. LE: Late exponential phase
d. S: Stationary phase

3.4.3 Fatty acid productions from Δ*actII-orf4*::Δ*fadD* mutants

From the previous report, deletion of *fadD* was reported to vanish β-oxidation because *fadD* initiates the catabolism by capturing fatty acids (Ford and Way, 2015). There are four *fadD* present in *S. coelicolor*. Among them, the one with highest transcription level, SCO6196, determined by average RPKM values of four time point sets, was selected for deletion. At this point, KASIII and BCDH double overexpression mutant was also constructed to maximize BCFA productions.

There was no differences in growths between WT, SCO_{dA}, and Δ*actII-orf4*Δ*fadD* (SCO_{dAF}) mutants. However, growths SCO_{dAF} overexpressing KASIII, BCDH or both decreased dramatically (Figure 3.6a). Decreases in total cell mass were more severe in SCO_{dAF} expressing larger genes in size. The average maximum cell mass of SCO_{dAF} was 9.35 g/L, but it decreased down to 7.89 g/L, 7.38 g/L, and 7.26 g/L in KASIII (SCO_{dAFK}), BCDH (SCO_{dAFB}), and KASIII + BCDH (SCO_{dAFKB}) overexpressing SCO_{dAF} mutants respectively. As reported in previous studies from other research groups (Banchio and Gramajo, 2002), *fadD* deletion almost vanished productions of ACT in the four strains (Figure 3.6b).


A
Dry Cell Weight (g/L, log10)
20
10
2
0
1
2
3
4
5
6
Time (Days)
WT::pIBR25
ΔactII-orf4::ΔfadD
ΔactII-orf4::ΔfadD::KASIII
ΔactII-orf4::ΔfadD::BCDH
ΔactII-orf4::ΔfadD::KASIII::BCDH
B
Actinorhodin A640nm (AU)
0.050
0.040
0.030
0.020
0.010
0.000
Day 3
Day 6
ΔactII-orf4::ΔfadD
ΔactII-orf4::ΔfadD::KASIII
ΔactII-orf4::ΔfadD::BCDH
ΔactII-orf4::ΔfadD::KASIII::BCDH

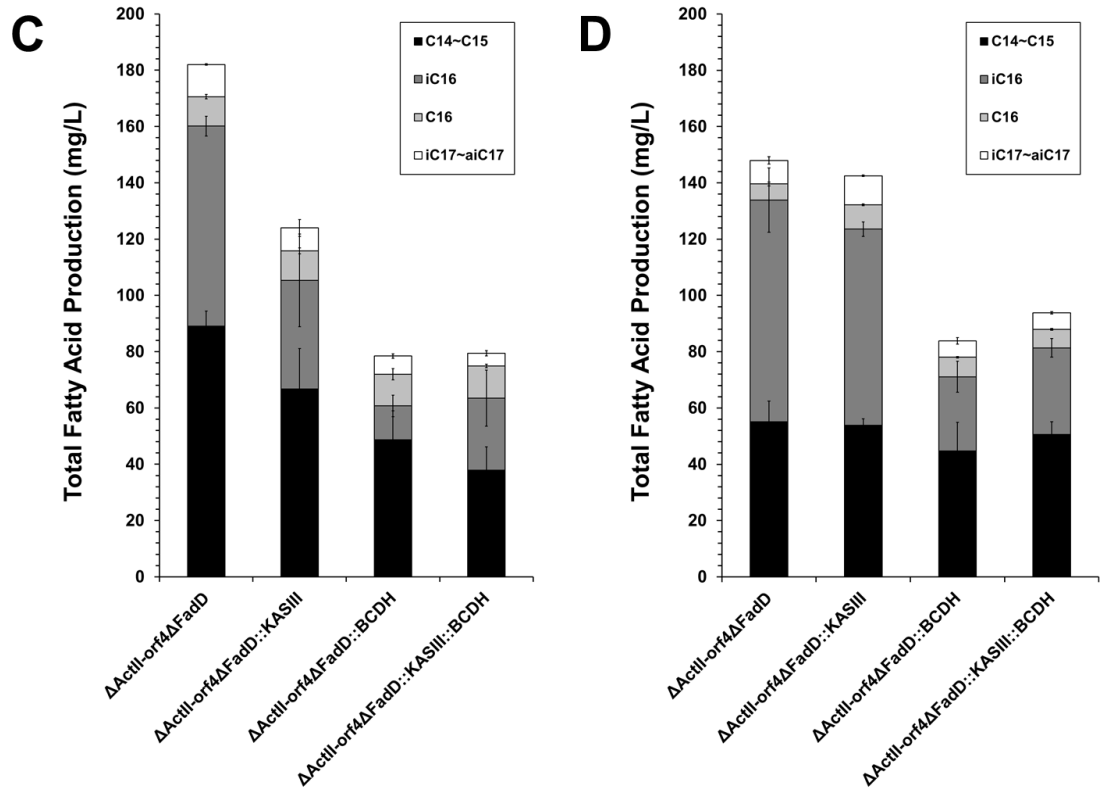
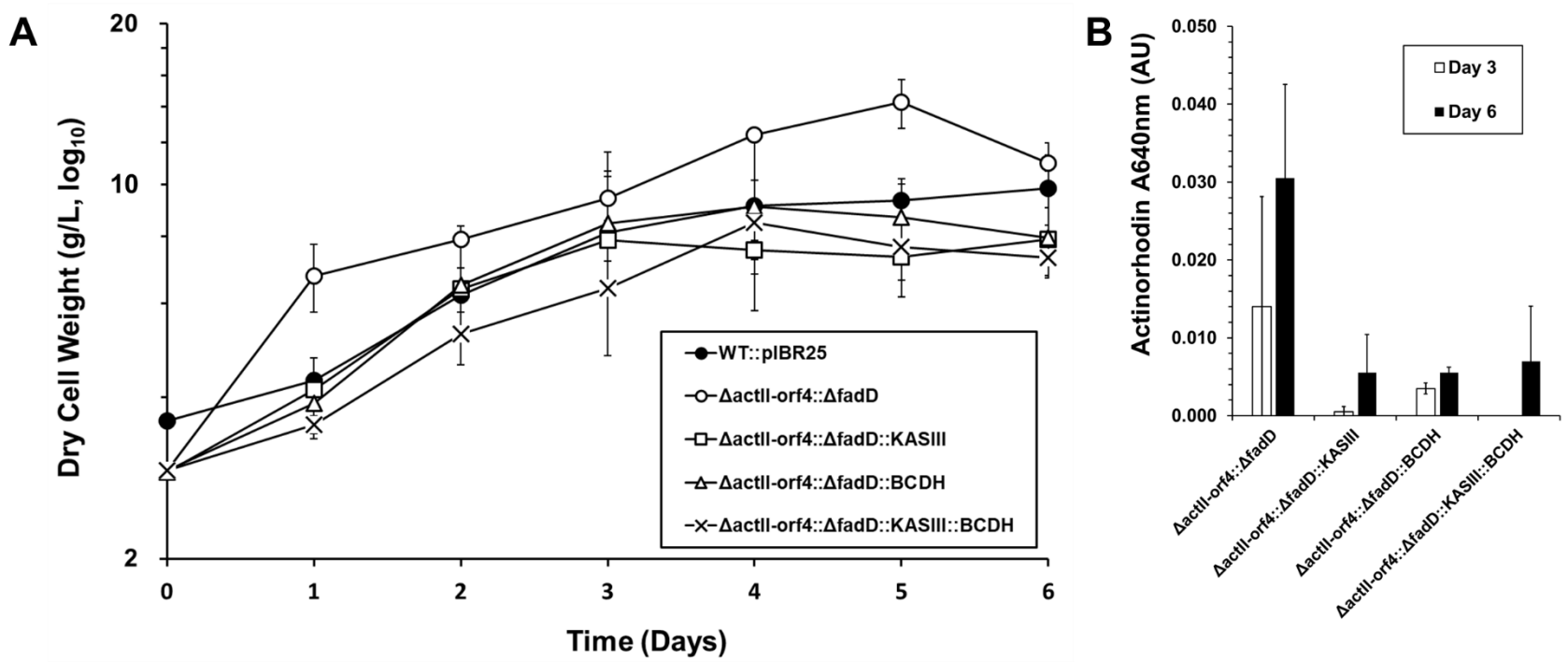


Figure 3.6. BCFA productions from SCO_{dAF} mutants. (a) Growth curves, (b) ACT productions, and BCFA productions on (c) day 3 and (d) day 6 of bacterial cultures.

FA productions in SCO_{dAF} increased from 85.05 ± 1.62 mg/L in SCO_{dA} to maximum of 181.0 ± 10.0 mg/L in SCO_{dAF} (Figure 3.6c and 3.6d). Unfortunately, as FA productions are closely related to the cell mass (Lu et al., 2008), total FAs decreased in the other three mutants. Previous reports indicate that repression in gene expressions of ACT gene cluster from deletion of *fadD* was the major reason for reduced productions of ACT, but also emphasize on losses of precursor supplementations, and altered carbon fluxes (Olukoshi and Packter, 1994). It may also have the same effects on the FA synthesis, since FA shares the same precursor pools with ACT. To solve such problems, we supplementationed leucine or vanillin.

First, as leucine supplementation was confirmed to increase BCFA productions by increasing growth rates, final concentrations of 2mM leucine was fed to the cultures of the four SCO_{dAF} mutants. Unfortunately, increases in growth rates or cell mass was not as distinctive as in SCO_{dA} (Figure 3.7a). There was no changes in ACT productions as well (Figure 3.7b). The maximum productions was with SCO_{dA} with 195.4 ± 24.8 mg/L, which was 14 mg/L increases. SCO_{dAFK}, SCO_{dAFB}, and SCO_{dAFKB} produced about 2 folds higher than those without leucine feeding. But it was not enough to produce more than SCO_{dAF} could (Figure 3.7c and d).


A
Dry Cell Weight (g/L, log10)
20
10
2
0
1
2
3
4
5
6
Time (Days)
WT::pIBR25
ΔactII-orf4::ΔfadD
ΔactII-orf4::ΔfadD::KASIII
ΔactII-orf4::ΔfadD::BCDH
ΔactII-orf4::ΔfadD::KASIII::BCDH
B
Actinorhodin A640nm (AU)
0.050
0.040
0.030
0.020
0.010
0.000
Day 3
Day 6
ΔactII-orf4::ΔfadD
ΔactII-orf4::ΔfadD::KASIII
ΔactII-orf4::ΔfadD::BCDH
ΔactII-orf4::ΔfadD::KASIII::BCDH

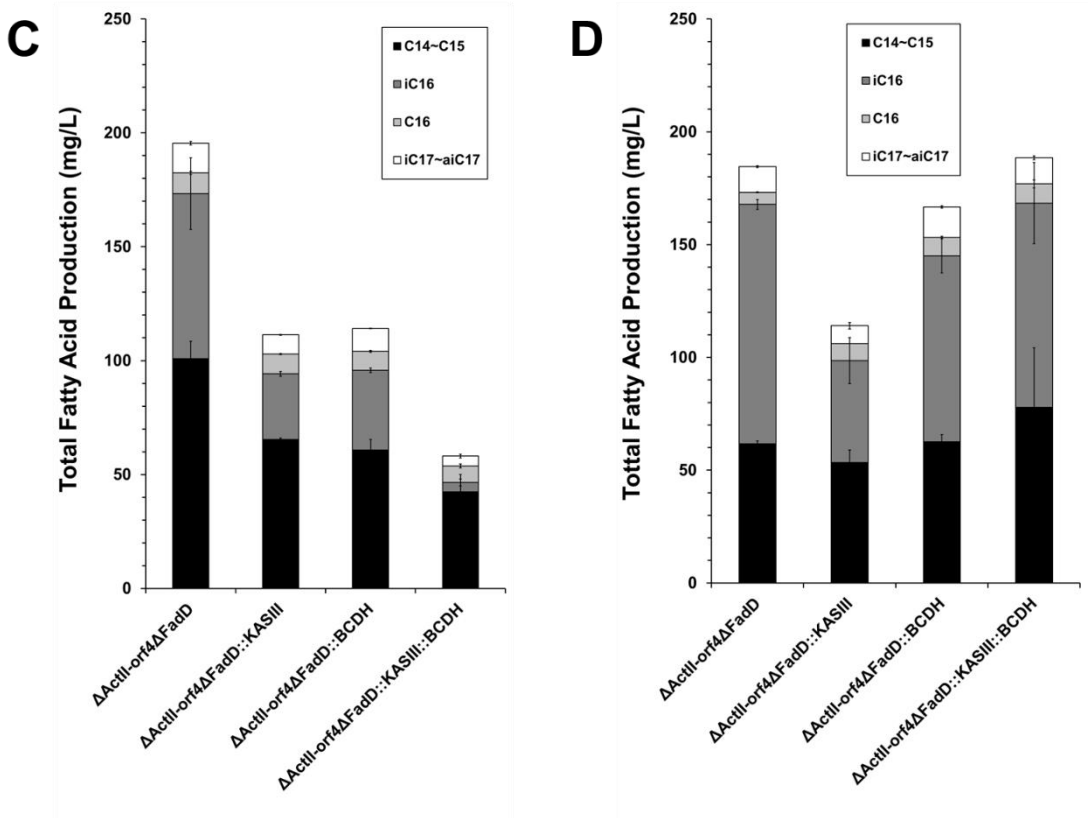


Figure 3.7. BCFA productions from SCO_{dAF} mutants with leucine supplementation. (a) Growth curves, (b) ACT productions, and BCFA productions on (c) day 3 and (d) day 6 of bacterial cultures.

Next, we supplemented vanillin to culture media to redirect acetyl-CoA fluxes into growth and fatty acids synthesis. Vanillin was known to reduce antibiotics productions in *Streptomyces* sp.. A previous report indicated vanillin affected cell morphology, development, and sporulation related genes, resulting in inhibited formation of mycelia and productions of antibiotics in *S. coelicolor* (Bhatia et al., 2016). In return, abolishment in antibiotics due to vanillin increased fatty acids contents. The previous report explained that decreases in ACT production possibly freed up extra acetyl-CoAs for FA synthesis.

Supplementations of final concentrations of 2 mM vanillin restored cell growths of SCO_{dAFK} to WT level, and the other two, SCO_{dAFB} and SCO_{dAFKB}, by about 1 ~ 2 g/L (Figure 3.8a). Cell masses were restored in higher quantities as the overexpressed genes were smaller in size. The maximum FA productions, which was also the highest throughout this study, were with SCO_{dAFK}, 250 ± 43.5 mg/L with 91.8% of BCFA contents, which was also the highest BCFA production titer among any other strain (Figure 3.8b and c). This suggested that growth optimization by controlling *fadD* expression rather than simple deletion, and / or other nutrient supplementations in KASIII and BCDH overexpressing SCO_{dAF} mutants would further increase FA productions in *S. coelicolor*.

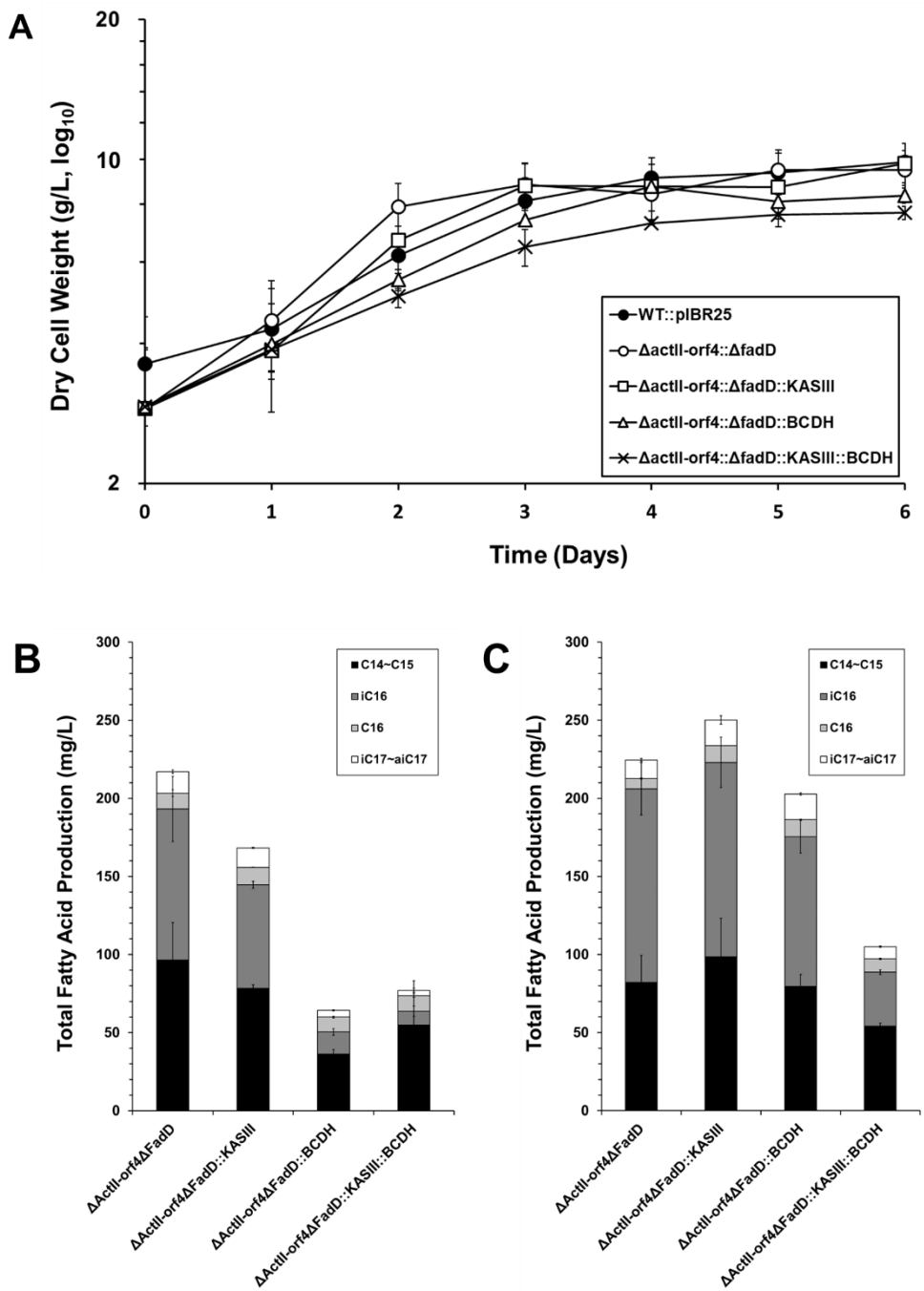


Figure 3.8. BCFA productions from SCO_{dAF} mutants with vanillin supplementation. (a) Growth curves, and BCFA productions on (b) day 3 and (c) day 6 of bacterial cultures.

3.4.4 Fatty acid productions from glucose

Another major metabolic pathway linked to fatty acid biosynthesis is glycolysis. Glycolysis is the major source of FA elongation units, acetyl-CoA and malonyl-CoA. For that reason, productions of SCFA in *E. coli* involved manipulations of glycolysis and FAS genes (i.e. pyruvate dehydrogenase and *fab* genes) (Janβen and Steinbuchel, 2014). BCAA synthesis pathway, branching from glycolysis, must be also manipulated for BCFAs, because they cannot be produced solely from glycolysis. Glycolysis does not yield any branched acyl-CoA precursors (Bentley et al., 2016; Haushalter et al., 2014). To produce BCFAs from glucose, we overexpressed *ilvBCD* (SCO_{dAilv}) of BCAA synthesis pathway in the Δ*actII-orf4* mutant. We also overexpressed NdgR (SCO_{dANdgR}), a well characterized positive regulator of BCAA synthesis pathway (Kim et al., 2015), in comparison to the overexpression of *ilvBCD*.

Overexpression of *ilvBCD* or NdgR both had positive effects on the production of BCFA, 125.7 ± 10.9 mg/L and 111 ± 48.1 mg/L respectively (Figure 3.9a and 3.9b). The titer was about the same as that from BCDH overexpression mutant. Difference is that anteiso-C15 and iC16 are equally increased in SCO_{dAilv} (Figure 3.9c). If growth problems with *fadD* deletion mutants could be resolved, it would be expected to further increase increase BCFA productions titer when *ilvBCD* expression was combined with that of KASIII and BCDH.

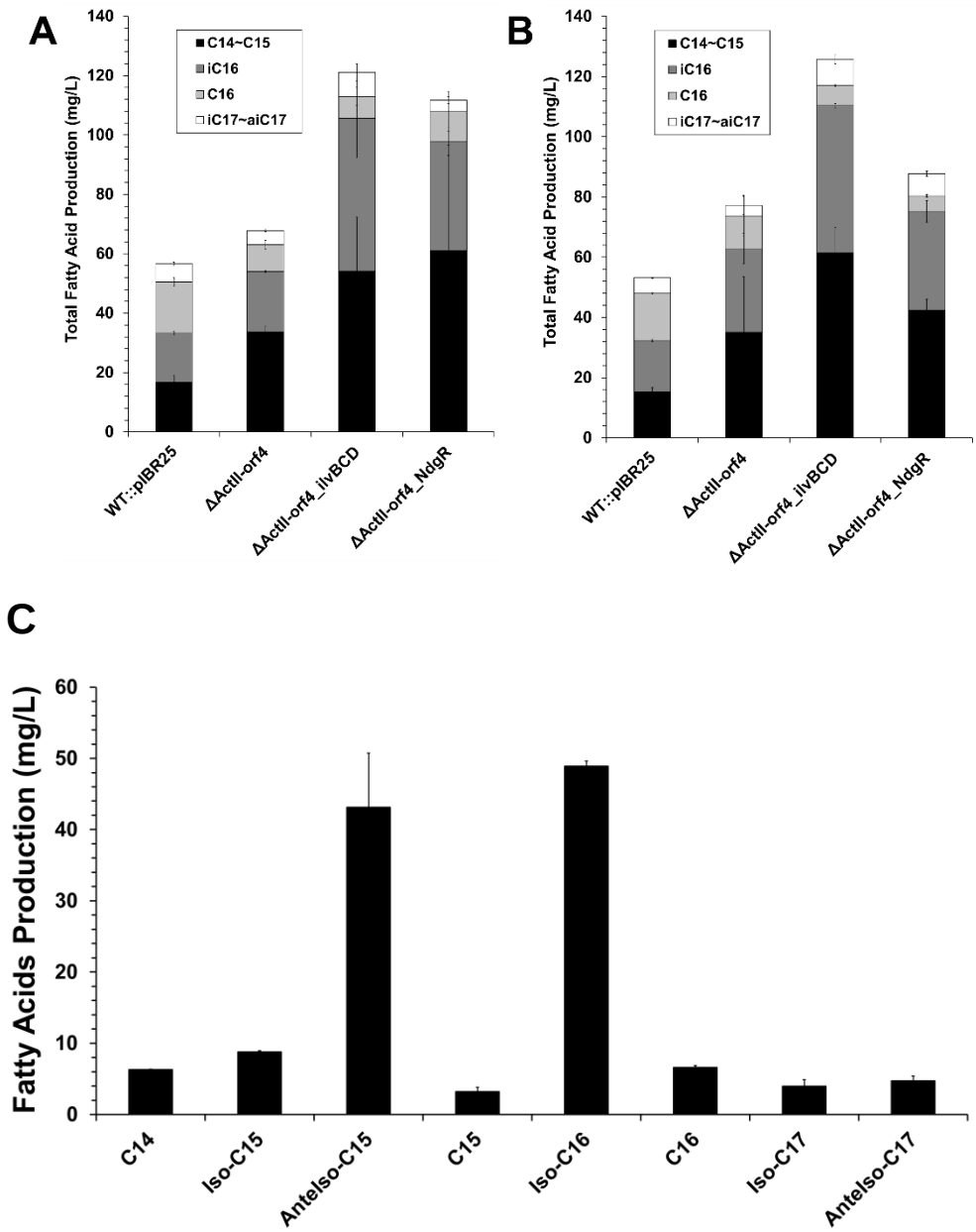


Figure 3.9. BCFA productions from SCO_{dABCD} and SCO_{dANdgR}. BCFA productions on (a) Day 3 and (b) day 6 of bacterial cultures. (c) FA contents of SCO_{dABCD}.

3.4.5 Fatty acid productions from *S. coelicolor* M1146

Since reducing ACT productions improved the FA productions in *S. coelicolor* M145 strain, we applied the same strategies on *S. coelicolor* M1146 (M1146) to further increase BCFA productions. M1146 strain has four different secondary metabolite gene clusters deleted (Coze et al., 2013), which are ACT, undecylprodigiosin (RED), calcium-dependent antibiotic (CDA), and a cryptic type I polyketide synthase (CPK). M1146 was originally constructed as a host for heterologous expression of other secondary metabolites (Gomez-Escribano and Bibb, 2010). But, with most of acyl-CoA competing pathways deleted, M1146 was also suitable for BCFA productions.

First, we compared total FA productions of the WT, SCO_{dA}, and M1146 strains. Growth of M1146 was not far different from that of the WT and SCO_{dA} (Figure 3.10a). On the other hand, production of FA in M1146 increased dramatically. On day 3 of cultures, FA productions of all three strains were about the same (Figure 3.10b), but on day 6, 184.5 ± 61.0 mg/L with 85 % of BCFA contents were produced in M1146 (Figure 3.10c). It was about 2.87 fold increases compared to that of the WT, and about 70 mg/L more than that of SCO_{dAB}. Anteiso-C15 and iC16 were the two major BCFAs in M1146, each counting for 52.9 ± 5.8 mg/L and 83.7 ± 20.3 mg/L respectively.

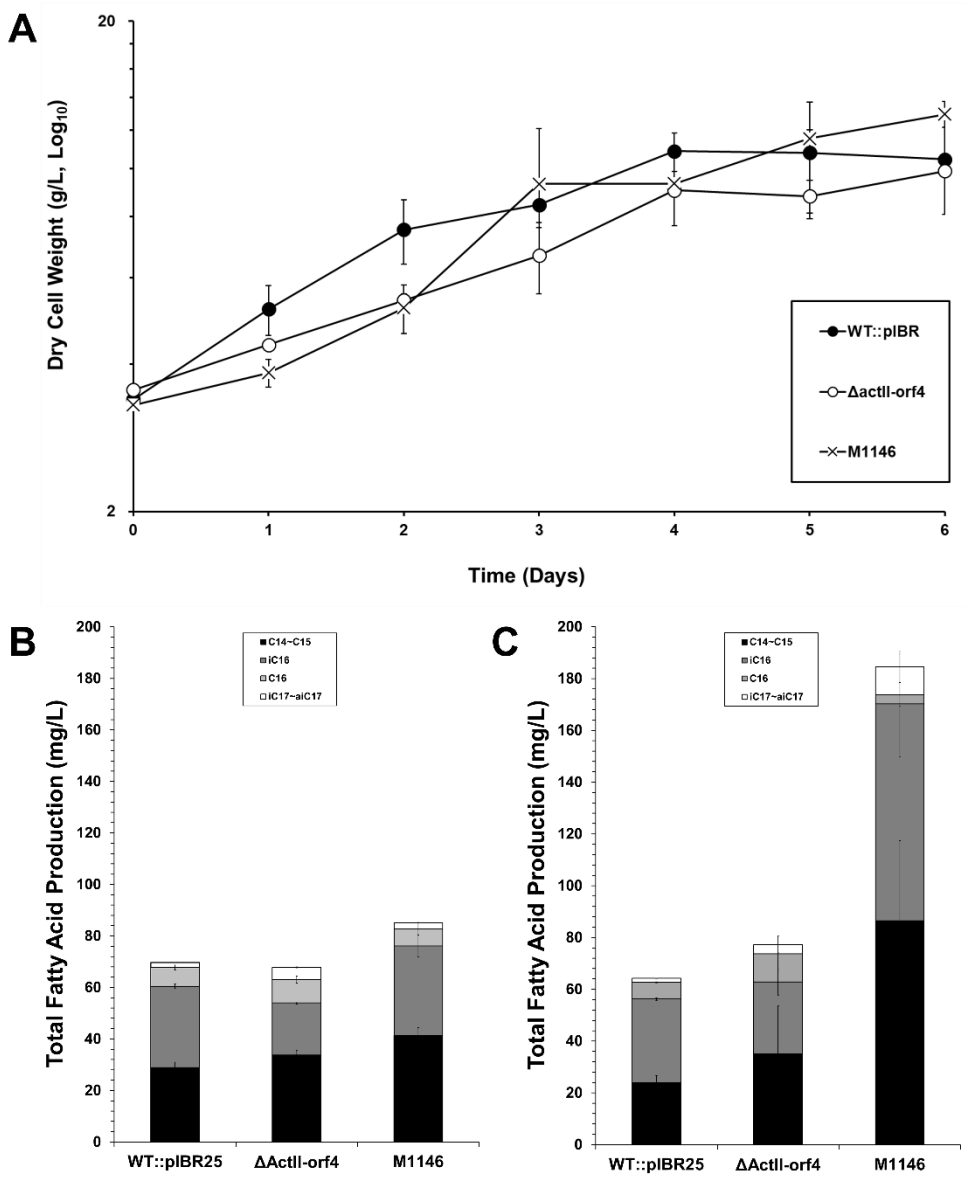


Figure 3.10. BCFA productions from the control, SCO_{dA}, and M1146. (a) Growth curves, and BCFA productions on (b) day 3 and (c) day 6 of bacterial cultures.

BCFA productions from M1146 strains was further increased by overexpressions of KASIII (M1146K), BCDH (M1146B), and both (M1146KB). Growths of M1146K, M1146B, and M1146KB decreased compared to that of the control, WT (Figure 3.11a). DCW was lower by average of 2 g/L in M1146K, M1146B, and M1146KB, with the lowest DCW in M1146B with 6.18 ± 0.02 g/L. Total FA productions were the highest in M1146KB, 190.4 ± 75.2 mg/L, with 87.3 % of BCFA. It was 1.18 folds increases compared to that of M1146, and 2.96 folds increases compared to that of the WT.

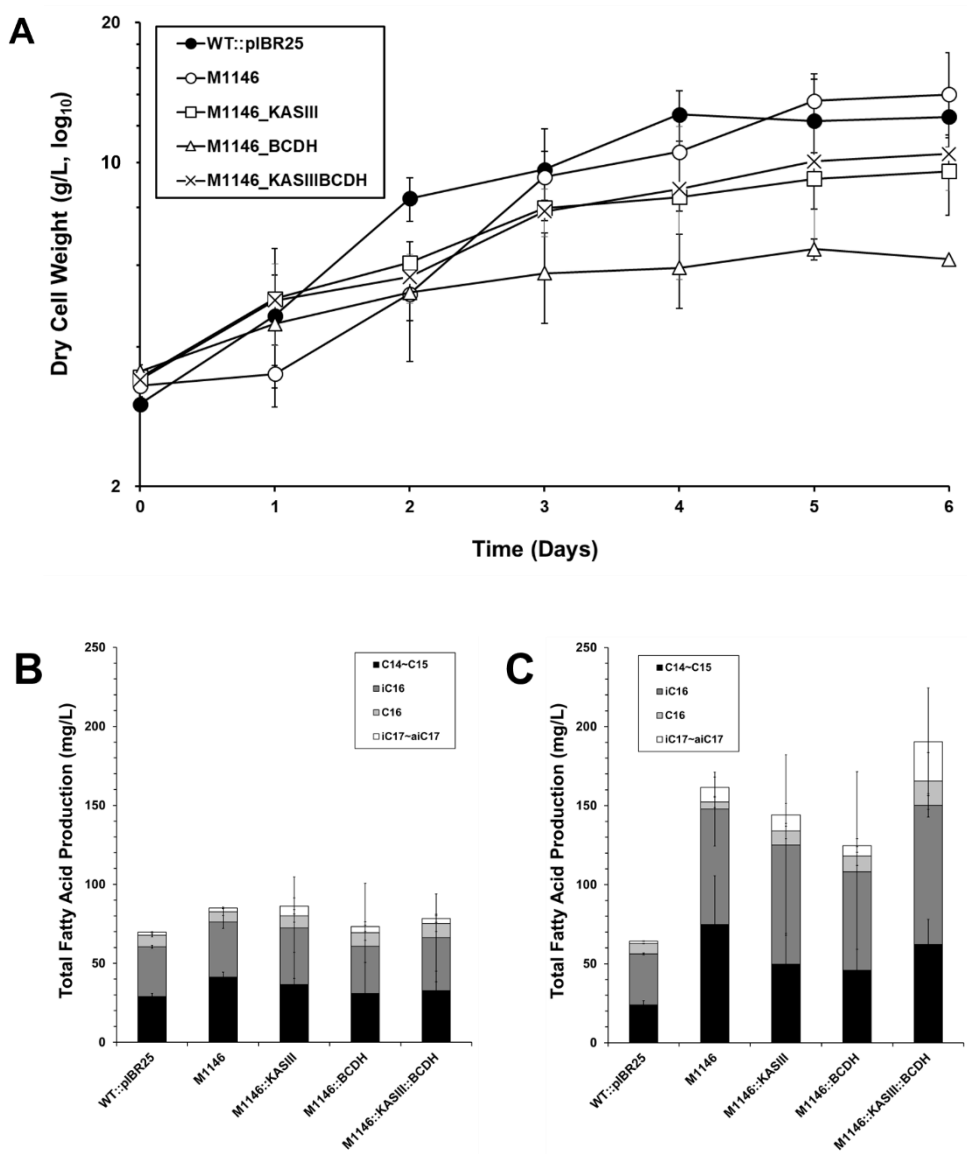


Figure 3.11. BCFA productions from the control, M1146, KASIII and BCDH overexpressing M1146 mutants. (a) Growth curves, and BCFA productions on (b) day 3 and (c) day 6 of bacterial cultures.

3.5 Conclusion

Taking advantages of morphological and biological features of *Streptomyces* sp., metabolic engineering within *S. coelicolor* was performed to increase productions of BCFA. We redirected acyl-CoA fluxes from polyketide towards FA productions by taking advantages of deleting secondary metabolite synthesis pathways. β-oxidation was also blocked by deletion of *fadD* so that accumulated FAs would not be catabolized back to acetyl-CoAs. KASIII and BCDH were overexpressed to increase initiations of fatty acids synthesis, and supplementations of BCFA precursors respectively. The maximum productions of FAs was 250 ± 43.5 mg/L from *S. coelicolor* Δ*actII-orf4*::Δ*fadD*::KASIII, with vanillin supplementations in media. Overexpression of *ilvBCD* also improved BCFA productions, that engineering central carbon metabolism and BCAA catabolism together would further provide a host strain, which is optimal for BCFA productions.

Chapter 4.

Production of Pikromycin Using Branched Chain Amino Acid Catabolism in *Streptomyces venezuelae* ATCC 15439

4.1 Abstract

Branched chain amino acids (BCAA) are catabolized into various acyl-CoA compounds, which are key precursors used in polyketide productions. Because of that, BCAA catabolism needs fine tuning of flux balances for enhancing the production of polyketide antibiotics. To enhance BCAA catabolism for pikromycin production in *Streptomyce venezuelae* ATCC 15439, three key enzymes of BCAA catabolism, 3-ketoacyl acyl carrier protein synthase III, acyl-CoA dehydrogenase, and branched chain α-keto acid dehydrogenase (BCDH) were manipulated. BCDH overexpression in the wild type strain resulted in 1.3 fold increases in pikromycin production compared to that of WT, resulting in total 25 mg/L of pikromycin. To further increase pikromycin production, methylmalonyl-CoA mutase linked to succinyl-CoA production was overexpressed along with BCDH. Overexpression of the two enzymes resulted in the highest titer of macrolide productions of 43 mg/L. However it accumulated and produced dehydroxylated forms of pikromycin and methymycin, including their derivatives as well. It indicated that activities of *pikC*, P450 monooxygenase, newly became a bottleneck in pikromycin synthesis.

Keywords: Methylmalonyl-CoA, Pikromycin, *Streptomyces venezuelae*

4.2 Introduction

Streptomyces species are soil dwelling bacteria producing vast numbers of polyketide secondary metabolites such as antibiotics and anti-cancer drugs, but such secondary metabolites are often produced in very small quantities (McKenzie *et al*., 2010). There are many efforts and studies to increase the productions of such secondary metabolites (Komatsu *et al*., 2010; Tang *et al*., 2017) in academia as well as fermentation industry. Among them, flux balance between fatty acids (FA) and polyketide (PK) biosynthesis is one general strategy to overproduce target molecules of interests, because FAs and polyketides share common acyl-CoA precursor pools (Brakhage, 2004; Kim *et al*., 2014). The commonality of using the same acyl-CoA substrates between fatty acid synthase (FAS) genes and polyketide synthase (PKS) genes would result a reciprocal relationship in their production yields to a certain extent at their high level productions, due to high similarities in their gene sequences and protein structures (Gajewski *et al*., 2017). In order to increase PK antibiotics productions, the precursors in FA biosynthesis should be redirected towards PK biosynthesis.

Dynamic controlling the flux of precursor pools towards FA biosynthesis and antibiotics production is very challenging, since FAs are essential components of lipid, consisting of 7 ~ 10 % of dry cell weight, as well as energy source for cell growth. One method to repress FA synthesis and increase PK productions is “redirection”, a strategy to reduce fluxes toward acyl-CoA consuming pathways. In a recent publication, addition of another antibiotics such as triclosan was newly suggested in *Streptomyces* sp. to achieve both successful repression of fatty acids

(Morvan *et al*., 2016), and enhancement of actinorhodin (ACT), erythromycin, and salinomycin productions from *S. coelicolor*, *Streptomyces erythraea*, and *Streptomyces albus*, respectively (Tanaka *et al*., 2017). However, this strategy has some drawbacks. First, addition of antibiotics such as triclosan are impractical and unreasonably expensive in real application. In addition, its clearance during purification generates another problem. As a result, controlling FAS gene expression may be a better strategy to reduce its FA synthesis. Another general, so called "pull" method to redirect acyl-CoA fluxes from FAS into PKS, is to overexpress entire PKS gene cluster (Nah et al., 2017), or by manipulations of transcriptional regulators (Tang et al., 2017). But PKS gene clusters are very bulky, which is hard to overexpress.

Enhancements of polyketide antibiotics productions can also be achieved with increasing entire precursor pools, so called "push" method, by enhancing the fluxes toward catabolism of key nutrients such as glucose (Ryu *et al*., 2006; Zabala et al., 2013). Glycolysis is a major source of acyl-CoA precursor productions (Chan *et al*., 2009; Dekleva and Strohl, 1998; Panagiotou *et al*., 2009). However, as there are limits to how much antibiotics could be produced from one nutrient source (Podojil *et al*., 1989; Laakel *et al*., 1994), metabolisms of other nutrients such as amino acids should also be considered. In case of PK, branched chain amino acid (BCAA) degradation was known to provide sufficient amounts of acyl-CoA precursors (Figure 4.1).

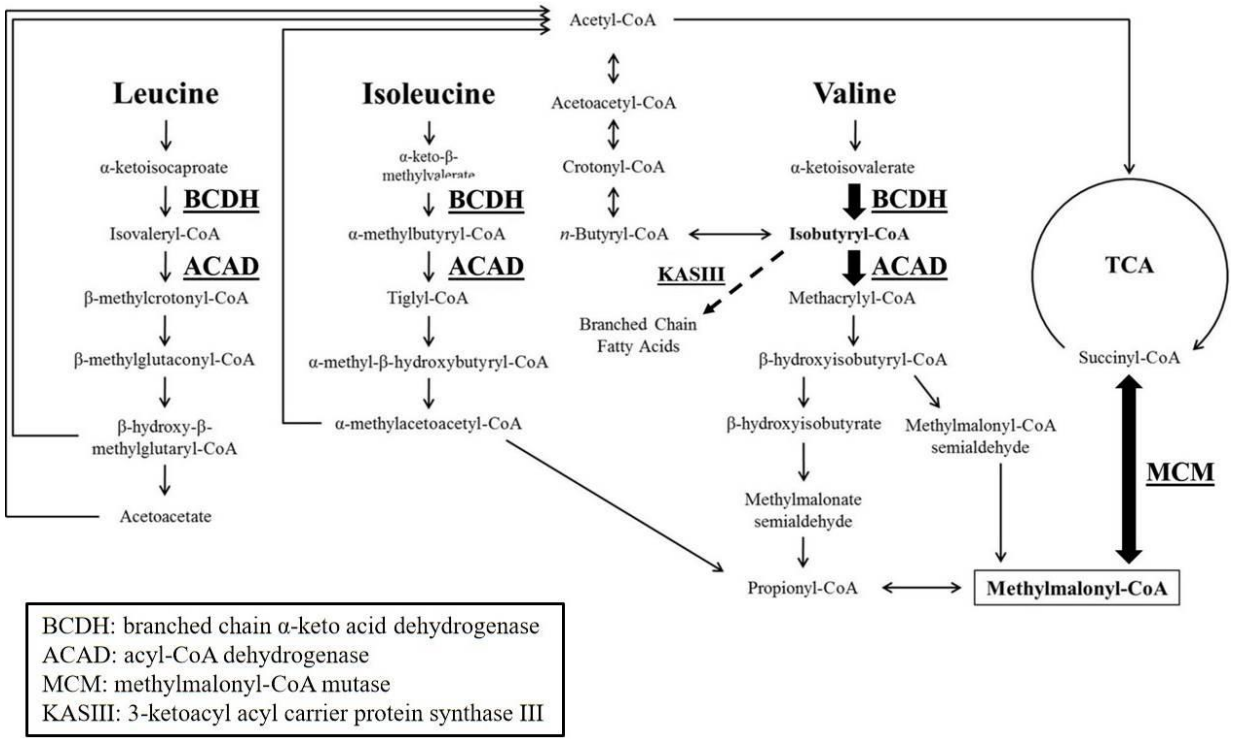


Figure 4.1. TCA cycle and valine catabolism pathway leading to methylmalonyl-CoA. Overexpression targets are indicated by bold arrow, and a repression target by dashed arrow. Methylmalonyl-CoA could be directly synthesized from succinyl-CoA or indirectly from valine. Isobutyryl-CoA acts as a hub, branching out to fatty acid synthesis or short chain acyl-CoA synthesis.

Branched chain α-keto acid dehydrogenase (BCDH) is a key common enzyme complex, participating in the catabolism of BCAA. Direct products of BCDH from valine, isoleucine, and leucine catabolism, are isobutyryl-CoA, α-methylbutyryl-CoA, and isovaleryl-CoA, respectively. The direct products of BCDH are further metabolized by acyl-CoA dehydrogenase (ACAD) (Pulsawat *et al*., 2007; Stirrett *et al*., 2009), or are used in synthesis of branched chain fatty acids (BCFA) (Wallace *et al*., 1995). It was previously reported that 50% of acetyl-CoA for ACT biosynthesis was supplied from the BCAA degradation pathway (Stirrett *et al*., 2009), and about 52 fold increase in ACT production was observed with BCDH overexpression mutant (Kim *et al*., 2014). Along with BCDH, ACAD also plays a key role in BCAA catabolism. ACAD pulls the fluxes of the three products of BCDH reactions away from fatty acid synthesis, and direct them to synthesis of the short chain acyl-CoA compounds, which are acetyl-CoA, propionyl-CoA, and methylmalonyl-CoA (Pulsawat *et al*., 2007; Zhang *et al*., 1999).

Despite of such importance of the two enzymes in the secondary metabolism, effects of BCDH and ACAD overexpression for antibiotics productions were not thoroughly investigated in *S. coelicolor* or *S. lividans* model strains, since ACT overproduced by the two strains do not use propionyl-CoA and methylmalonyl-CoA, but only use acetyl-CoA and malonyl-CoA as their precursors (Ryu *et al*., 2006). We were interested how BCAA catabolism would affect pikromycin (PKM) productions in *S. venezuelae*, as PKM is biosynthesized using six methylmalonyl-CoAs and one malonyl-CoA (Wilson *et al*., 2001; Phelan *et al*., 2017). A simple solution to investigate effects of BCAA catabolism on the PKM production is to make net fluxes

of precursor pools positive by overexpression of key enzymes, which results enhanced its biosynthesis.

In this study, we applied and combined different methylmalonyl-CoA synthetic routes for PKM biosynthesis in *S. venezuelae* ATCC 15439. As overexpression of PKS gene cluster may result in the limitations of acyl-CoA precursor pools, we wanted to set up a guideline for selection of optimal metabolic pathways to overproduce PK antibiotics (for example, PDH for ACT productions in *S. coelicolor*). Using BCAA catabolic pathway, we applied three different strategies for PKM overproduction: (1) "redirection" of acyl-CoA fluxes by repression of β-ketoacyl acyl carrier protein synthase III (KASIII), competing pathway, (2) "push" by overexpression of BCDH, and (3) "pull" by overexpression of ACAD to increase BCAA catabolism. Methylmalonyl-CoA mutase (MCM) was overexpressed along with BCDH to combine methylmalonyl-CoA synthesis from both BCAA catabolism and tricarboxylic acid (TCA) cycle.

4.3 Materials and Methods

4.3.1 Bacterial strains, plasmids, and culture conditions

All strain information and plasmids used in this study are listed in Table 4.1. *E. coli* strains were cultured in Luria-Bertani (LB) broth (Becton Dickinson, USA) with 0.1 mg/mL ampicillin or 50 μg/mL apramycin when needed.

Table 4.1. A list of bacterial strains and plasmids used in this study

Strains or Plasmids	Descriptions	References
Streptomyces strains		
Streptomyces venezuelae ATCC15439	Wild type strain	Yi *et al*., 2015
E. coli strains		
DH5α	A host for gene manipulations	Invitrogen
ET12567	A host for transfering plasmids into *Streptomyces* sp.	ATCC
Plamids		
pIBR25	SCP2* origin, ampicillin and thiostrepton marker	Yi *et al*., 2017
SuperCos-1	a cosmid vector, pUC origin, ampicillin and neomycin marker	Agilent Technologies
pSET152	*E. coli* - *Streptomyces* sp. shuttle vector, pUC18 origin, ΦC31 integrase and attP site, apramycin marker	Swiatek *et al*., 2013
pIBR25KASIII	pIBR25 containing *fabH* in *Pst*I/*Xba*I sites	this study
p1220KASIII	SuperCos-1 containing *fabH* with a promoter of SCO1214 and 5' UTR of SCO2078	this study
pIBR25BCDH	pIBR25 containing *bkdA1B1C1* in *Xba*I / *Eco*RI sites	this study
pIBR25ACAD	pIBR25 containing *acdH* in *Bam*HI / *Pst*I sites	this study
pIBR25MCM	pIBR25 containing *mutA2* in *Eco*RI / *Hind*III sites	this study
pIBR25BM	pIBR25 containing *bkdA1B1C1* in *Xba*I / *Eco*RI sites and *mutA2* in *Eco*RI / *Hind*III sites	this study
pIBR25PikC	pIBR25 containing *pikC* in *Pst*I / *Xba*I sites	this study
pSET152PikC	pSET152 containing ermE*P and *pikC* in *Eco*RV / *Xba*I sites	this study

R2YE complex medium was made following standard protocols [9], and used for cultures of *S. venezuelae* strains. R2YE, Difco Nutrient Agar (DNA) media (Becton Dickinson, USA), and Mannitol Soya flour Agar (MSA) media made by standard protocols were used for conjugation of cloned plasmids such as p1220FabH and pSET152PikC, and *S. venezuelae* spore preparations. 8 μg/mL thiostrepton and 50 μg/mL apramycin were used for selection of *S. venezuelae* mutants and conjugates. Filter sterilized 100 mM valine was added to *S. venezuelae* cultures at the time of inoculation to study the effects of valine on pikromycin production. Cell growth was also observed by UV absorbance at 600 nm using Multiskan spectroscopy (Thermo Fisher Scientific, USA), and cell pallets after pikromycin extractions were dried in an oven at 60 ℃ for dry cell weight (DCW) measurements.

E. coli DH5α was cultured at 37 ºC. *E. coli* ET12567 and *Streptomyces* strains were cultured at 30 ℃, both with 200 rpm shaking in Lab Companion SK-71 Bench top shaker (Jeio Tech, South Korea). 200 mg of wet weighted cells from 50 mL seed cultures grown overnight were inoculated into 50 mL R2YE media in 250 mL flasks.

4.3.2 Strain construction

All primers and their sequences are listed in Table 4.2. PCR amplifications were performed with LA-taq DNA polymerase with GC buffer (TaKaRa, Japan) using Thermal Cycler Dice (TaKaRa, Japan). FastDigest (Thermo Fisher Scientific, USA) restriction enzymes were used for digestions of PCR amplified DNA fragments.

Table 4.2. List of primers and their sequences

Plasmids	Sequences
p1220FabH	
*fabH*_Up_Forward	AATTAACCCTCACTAAAGGGATCCTCGATCT GCTCCTGGAGTGCCAT
*fabH*_Up_Reverse	GAAGCAGCTCCAGCCTACATCGGACGTGCGG AACATCGTGTAC
*aac(3)-IV*_Forward	TGTAGGCTGGAGCTGCTTC
*aac(3)-IV*_Reverse	ATTCCGGGGATCCGTCGACC
$P_{SCO1214}$_Forward	AACTGCAGGTCGACGGATCCCCGGAATCTCG CCCAGGTCCGCTTCG
$P_{SCO1214}$_Reverse	TGTGAAGGCACTCCCGTGAGGCCCCGCCACC CTAGCGGCAT
$R_{SCO2078}$_Forward	ACACTTCCGTGAGGGCACTCCG
$R_{SCO2078}$_Reverse	GACCTGTGCTTCCCTCTCCCCT
*fabH*_Forward	TTCAGGGGAGAGGGAAGCACAGGTCATGAC GGGCACTCGCATCGC
*fabH*_Reverse	AGTCAACAAAAAGCAGACGTATCTAGAGGCC TTCGGCGCCGAGCT
SuperCos_Forward	TCTAGATACGTCTGCTTTTTGTTGACTT
SuperCos_Reverse	GGATCCCTTTAGTGAGGGTTAATT
KASIII	
Forward	ATTATCTGCAGGAAGGATGCGGTTACACGGT CCGAGGAGGGAGCCACCGATGTCGAAGATC AAGCCCAGCAAG
Reverse	ATTATTCTAGATGATCCGGCACGGAGTGCCT AG

BCDH	
Forward	ATAATCTAGACCCACCCTTGTGCGTCCG
Reverse	ATTATGAATTCTCAGGCCCAGCTGATCAACCGCT
ACAD	
Forward	ATTATGGATCCAGCCCTCTCCCACCAGGA
Reverse	ATATACTGCAGTCGGAGCCTCAGCCCACCA
MCM	
Forward	ATAATGAATTCTAGATGACGGGTAGTTCGATG
Reverse	ATATAAAGCTTGTCAGAACGCGTCCGAAGG
pikC	
Forward	ATAATCTGCAGCGGAAGAGTACGTGTGAGAAGT
Reverse	ATTATTCTAGACTTCCGTGACAAGGAGTCGTAATG
ermE*P_*pikC*_Forward	ATAATGATATCTTGTAAAACGACGGCCAGTGAATTAATTC
qPCR Primers	
16S rRNA_Forward	CCTTCGGGTTGTAAACCTCTTTCAGCA
16S rRNA_Forward	CAACACCTAGTTCCCAACGTTTACGGC
*fabH*_Forward	CGAAGGTGCTCACCAACC
*fabH*_Reverse	CAGACCACGTTCAGGTCCAT

Promoter and 5’ UTR for repression of KASIII were selected from *S. coelicolor*, which was described in the previous study [38]. Three promoters were tested including $P_{SCO4505}$, $P_{SCO4808}$ and $P_{SCO1214}$. Promoter of SCO1214 and 5’ UTR of SCO2078 were selected to repress KASIII transcription. A cosmid of KASIII carrying promoter of SCO1214 and 5’ UTR of SCO2078 was constructed using SuperCos-1 with Gibson Assembly (New England BioLabs, USA) following a procedure of the manufacturer. Overexpression plasmids of KASIII (SCO2388, *fabH*), BCDH (SCO3815-3817 *bkdA1B1C1*), ACAD (SCO2279 *acdH*), and MCM (SCO4869 *mutA2*), were constructed by conventional cloning methods with restriction enzymes. *pikC* overexpression plasmid was constructed in the same manner. For the integration of ermE*P *pikC* into the host chromosome, *pikC* was first inserted into pIBR25 plasmid, resulting pIBR25PikC. ermE*P and *pikC* were PCR amplified altogether using pIBR25PikC as a template, and then inserted into pSET152.

Plasmids were conjugated to *S. venezuelae* using *E. coli* ET12567, and all other overexpression plasmids were transformed into *S. venezuelae* using protoplast transformation methods (Kieser et al., 2000).

4.3.3 Quantification of KASIII transcription level

mRNA of *S. venezuelae* mutants were extracted with RNeasy Mini Kit (Qiagen, Germany). cDNAs were synthesized from 1.5 µg of the extracted mRNAs using M-MLV reverse transcriptase (Promega, USA). mRNA extraction and cDNA synthesis were performed according to standard protocols provided by the manufacturers. 16S

rRNA was used as an internal standard for normalization of Real Time PCR (qPCR) data, and calculation of relative KASIII expression levels. LightCycler 480 (Roche, Switzerland) was used for qPCR analysis. TOPreal qPCR 2X PreMIX containing SYBR Green with low ROX (Enzynomics, Korea), and standard qPCR settings from the SYBR manufacturer were used for the analysis.

4.3.4 Pikromycin extraction and quantification

On day 3 of *S. venezuelae* cultures, 40 mL culture broths were harvested and supernatants were collected by centrifugation at 2,840 X g for 10 min. PKM was extracted from the supernatants using two volumes (80 mL) of ethyl acetate. After the extracts were dried with a rotary vacuum evaporator, the remaining part was dissolved with 1.8 mL methanol. Leftover cell pellets from the harvested 40 mL cultures were used for calculations of DCW.

PKM, methymycin (MTM) and their derivatives were quantified using 20 µL of cell extracts by high-performance liquid chromatography (HPLC) YL-9100 (Younglin, Korea). A C_{18} reverse phase column (Waters, USA) with a linear gradient system at flow rate of 1 mL/min was used as described in our previous study (Yi et al., 2015). PKM, MTM, and their derivatives corresponding peaks were analyzed and confirmed by TSQ Quantum Access Max Triple Quadrupole Mass Spectrometer, (TSQ-MS, Thermo Fisher Scientific, USA) using 5% ACN with 0.28% ammonium hydroxide and 5mM ammonium acetate.

4.4 Results and Discussion

4.4.1 Impact of valine supplementation on pikromycin production

Catabolism of BCAA results in various low molecular weight acyl-CoA (Figure 4.1). Valine can possibly produce three, acetyl-CoA, propionyl-CoA, and methylmalonyl-CoA. Thus, we focused on effects of valine supplementation on PKM production. To examine an optimal level of valine feeding in batch flask cultures of *S. venezuelae*, R2YE complex media containing five different final valine concentrations (i.e. 2, 4, 6, 8, and 10 mM) were compared for cell growths and PKM production preliminarily. Valine supplementation decreased total cell mass at higher concentrations (Figure 4.2a). The maximum pikromycin titer, 22.6 mg/L, was observed at 2 mM valine, which was 1.2 folds and 3 mg/L more than that without valine supplementation (Figure 4.2b).

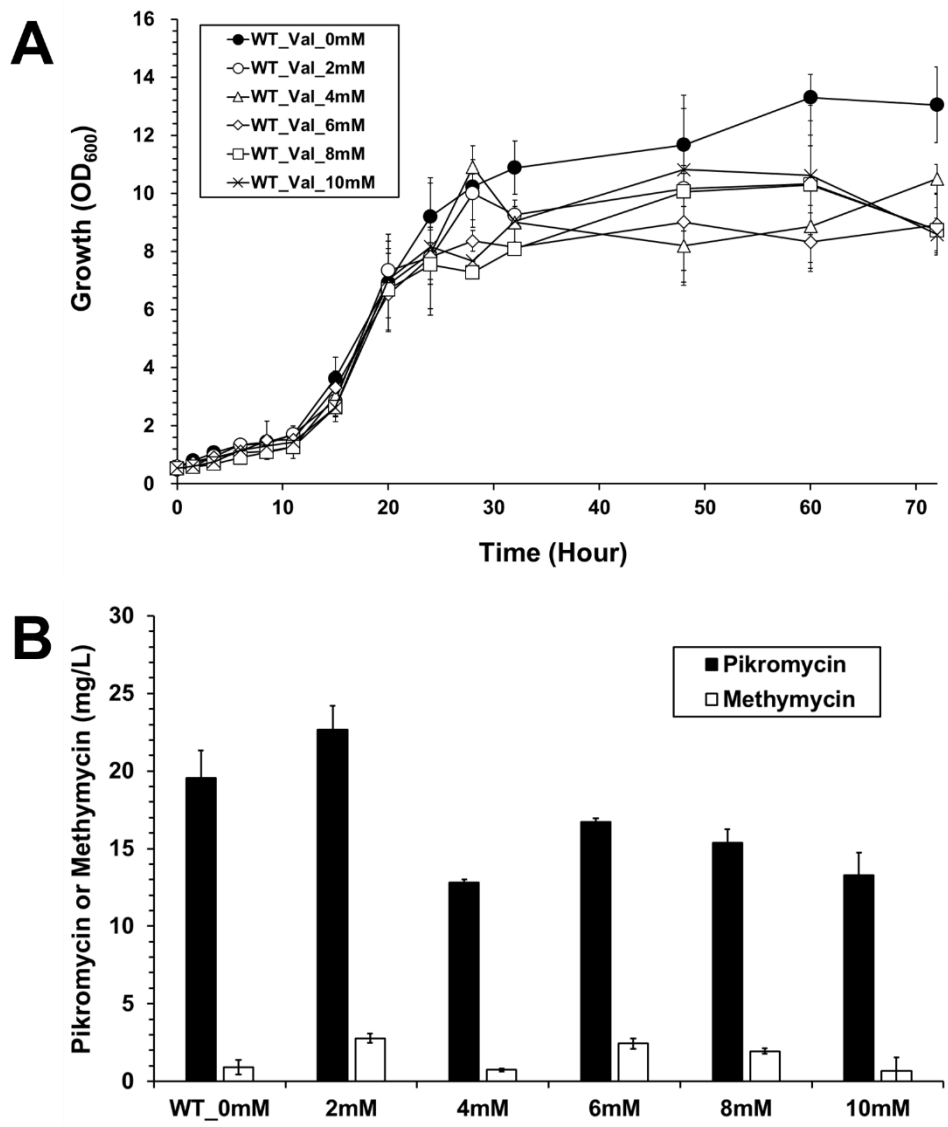


Figure 4.2. (a) Growth and (b) pikromycin and methymycin productions with valine supplementations. Increasing valine supplementation over a certain concentration reduced cell growths, and consequently also reduced antibiotics productions.

A reduction of PKM production with increasing valine concentrations may be explained by roles of valine. Valine supplementation was previously reported to activate expressions of pyruvate dehydrogenase, but repress glycolysis, inhibiting pyruvate formation in *Bacillus subtilis* (Ye *et al*., 2009). In *Streptomyces ambofaciens*, excess valine resulted in ammonium formation and accumulation, reducing cell mass and spiramycin production yields (Lounes *et al*., 1995). Reduced cell growth in *S. venezuelae* at higher valine concentrations may have reduced pikromycin productions as well. In addition, valine is an activator of a global regulator *codY*. *codY* is activated by access valine, and represses formation of acetyl-CoA from acetate, isocitrate from citrate, and glutamate from 2-oxoglutarate (Katz and Brown, 1989; Sonenshein, 2007; Gopalani *et al*., 2016). Additionally, biosynthesis of BCAAs are subjected to many other regulations such as amino acid metabolisms (i.e. glutamate and arginine), and Lrp family transcriptional regulators (Sonenshein, 2007), so that an optimal valine feeding concentration is required. Since 2mM valine supplementation resulted in some increases in PKM production, it appeared that there were still rooms to utilize BCAA degradation pathways by modulating metabolic enzymes, and / or regulators to increase PKM production.

Valine feeding alone may not result in great increase in pikromycin production. Since effects of BCAA catabolism on polyketide antibiotics were not thoroughly studied so far, we have decided to further examine their relationships. Our valine supplementation experiment suggested that only a small portion of valine was actually converted into methylmalonyl-CoA and malonyl-CoA. Thus, it appears that there are still rooms to utilize BCAA degradation pathways by modulating

metabolic enzymes and/or regulators to increase the metabolite fluxes into pikromycin precursor.

4.4.2 BCAA catabolism and pikromycin productions

When BCAAs are catabolized into acyl-CoA compounds via BCDH, they are used for either synthesis of BCFA or polyketide secondary metabolites. KASIII for BCFA (Kaneda, 1991), and ACAD for short chain acyl-CoAs (Zhang *et al*., 1999) are responsible for the two pathways, respectively. Since FAS and PKS compete for the same substrates, and reduction of FA synthesis was effective to improve PK productions (Tanaka et al., 2017), reduction of KASIII was first performed for PKM. We aimed to reduce acyl-CoA flux into FA synthesis by controlling KASIII gene expression, replacing a promoter and 5' UTR of KASIII with the ones developed from our previous study (Yi *et al*., 2017).

Promoter of SCO1214 ($P_{SCO1214}$) was included on top of the two previously developed promoter and 5' UTR sets from *S. coelicolor*. Total three sets of promoters and 5' UTRs were inserted and replaced the WT promoter and RBS of KASIII in the chromosome. The three sets are $P_{SCO4505}R_{SCO0641}$, $P_{SCO4808}R_{SCO2078}$, and $P_{SCO1214}R_{SCO2078}$, and they were chosen for low expression levels of β-$_D$-glucuronidase (Table 4.3).

Table 4.3. Sequences of the promoters and 5' UTRs used for KASIII repression

	Sequence
$P_{SCO4505}$	TTCTTGAGGAATTCGACCAGGGTGACGGGACCGATGTCGAGATCACCG
	CGCACCAGCTGCTCGCTGAGCTTCTCCGGGGTGTCCTTCGTCAGCTCGA
	AGTCGAGGAGCGTGCCTGTTCTCGCGAGCCCCCAGTACAGGGGCAGGC
	AGTTCAGGAACTGGATGTGGCCGACACGCGGCCGGGTGCGAGAATTGT
	CCACATCGCGAGGCTAGCCCTCCCCCGGGAACCGCCCCTCGCCGACCC
	CCGGCGTCAGCCGAACGGCGCAGGTGTTCAAACGTCCGGGTGAAGTGA
	TCTTGACCTCTGTTGCGCTCGGGGGCCTGCGTGCTAGGCTCACAGC
$P_{SCO4808}$	CAGACCCCGCGGGGCGGCAGGCCCCGGACGGCCGAAAGCCGTCGGCCT
	CCGTCCGGCCGAAACCCCGCGGTCCGGTCCGGCGGGTCCCCATTGGGG
	TGGGGACCAGCCGGACCGCACCCCGGCGGACCGCATCCGTGCGGCCCG
	CCGGGAGCCCACGGGCCTCACCGGGTCAACCCCAGCCCGGTGAGGCCC
	TTCACCCGATCTGCGGAGCGCCCCGTGTCCACCTCACGCACGGCACCGC
	GTGCCAGTGAGCGCGGCATCATGTGACAGGCATCACCGCTCAGGTGTG
	ACCCACGATTTAGAGACCCCCGGGAAGCGGCGATAACCTGCGAGAC
$P_{SCO1214}$	CTCGCCCAGGTCCGCTTCGGCAACGGCAACGGCACGGGGGACGGCACG
	GAGGGCGCGTACAACGACACGGTCTTCGGCACCTACATGCACGGCCCC
	GTGCTGGCCCGCAACCCGCTGATCGCGGACCTGCTGCTCAAGCTGGCCC
	TCGACGTGAACGCGCTGCCGCCGACCGACGACCGCTGGTACGAGGCGC
	TGCGCAACGAGCGCATCGCGGCCGCGCAGCAGCCTGCCTGACCCTGGC
	GGGGCGGGCCCGTCTGATGCCCCGTCCGCACAGGTGAGCGGACTCGTC
	CAGTAGGCGGACGCACGGTTCGGTCCCGCCCCCCGATGCCGCTAGGGT
	GGCGGG
$R_{SCO0641}$	AGGCACGCACACACGGAACACGACGGACACGACGGACACGACGAAGG
	AGAACGCA

$R_{SCO2078}$	ACACTTCCGTGAGGGCACTCCGGTGATCATGAATCTCACTGAGATGGAT
	GACACAGATGCGAAGCGACTTGTCGACTTTGCGGCCGGTTTGGTGTTTG
	GTCTTCACGGCAGTATCGAGCGGGTGACGCAGAAGGTGTTCCTGCTGTC
	TCCTGCTAACGTCGATGTCACGGCGGAGGACAAGGCCCGTATCGCAGA
	GGGCGGGTTCTTCAACCAGAGCTGAGACGCACTACCGGTACAGAGCAA
	GAACACGGGCCCGGGACAGGGCCCGAGAGATGGTTCAGGGGAGAGGG
	AAGCACAGGTC

Nonfunctional *S. coelicolor* promoters and 5' UTRs in *S. venezuelae* were easily screened out by live and death assay, because without KASIII, cells could not grow (Li and Reynolds, 2005). $P_{SCO1214}R_{SCO2078}$ was selected to repress KASIII transcription. All the names of the promoters and 5' UTRs with their sequences are listed in Table S2. According to data of qPCR measurement, successful 24 folds repression of KASIII transcription was achieved using $P_{SCO1214}$ and $R_{SCO2078}$ ($SVEN_{1220KASIII}$) compared to that of the wild type (Figure 4.3a).

The growth of $SVEN_{1220KASIII}$ slightly decreased compared to that of the wild type, measured by cell optical density (OD) (Figure 4.3b), which was also confirmed by DCW measurement (i.e. $DCW_{1220FabH}$ = 6 g/L, which is about 83% of the control (Figure 4.3c)). In contrast, pikromycin titer of $SVEN_{1220KASIII}$ decreased more dramatically, by almost a half (Figure 4.3d). Therefore, we realized that KASIII repression is not a good strategy for enhancing PKM production at this moment. It would require more thorough understanding of regulating KASIII expression.

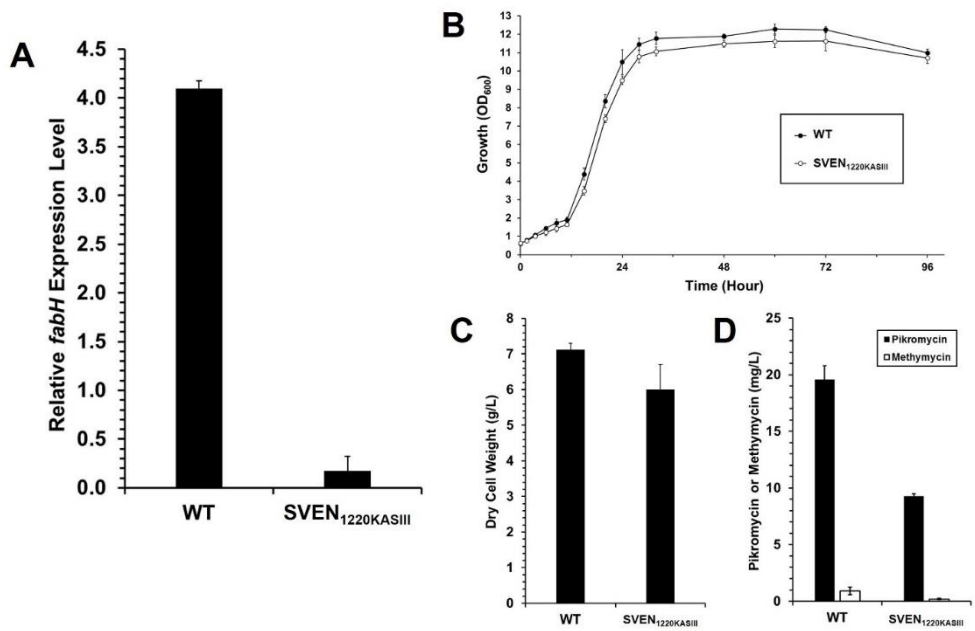


Figure 4.3. Expression of KASIII, growths, and pikromycin productions from KASIII repression mutatnt. (a) relative expression levels of KASIII normalized by 16s rRNA expression level, (b) growth by O.D., (c) DCW in g/L, and (d) antibiotics productions in mg/L from WT and KASIII repression mutant.

To increase metabolic flux towards methylmalonyl-CoA synthesis, we used push and pull strategy by overexpressing BCDH ($SVEN_{bkd}$) and ACAD ($SVEN_{acdH}$). There were little variations in cell growths between the three overexpression mutants, but they were almost identical, about 7 g/L DCW (Figure 4.4a). Similar to the case of valine supplementation, production of PKM was highly correlated with the cell growth, and the maximum concentration of pikromycin was 25.02 mg/L, which was 1.28 fold increases of that from the WT (Figure 4.4b). It was unexpectedly small increase in the antibiotics productions, when compared to the data of our previous study in *S. coelicolor* (Kim et al., 2014). It may be explained by chances of methylmalonyl-CoA synthesis from the BCAA catabolism.

Theoretically, only acetyl-CoAs could be produced from leucine. Either one acetyl-CoA or one propionyl-CoA could be produced from isoleucine, and either one acetyl-CoA, one propionyl-CoA, or one methylmalonyl-CoA could be produced from valine (Zhang *et al*., 1996; She *et al*., 2013). Therefore, the building block of PKM, methylmalonyl-CoA, would be generated in smaller chances in general compared to acetyl-CoA for ACT. But, since acetyl-CoA can be converted into methylmalonyl-CoA via succinyl-CoA through TCA cycle, perhaps the rate-determining step (RDS) of the synthesis of PKM may be in a different step or in some gene regulation of the pathway.

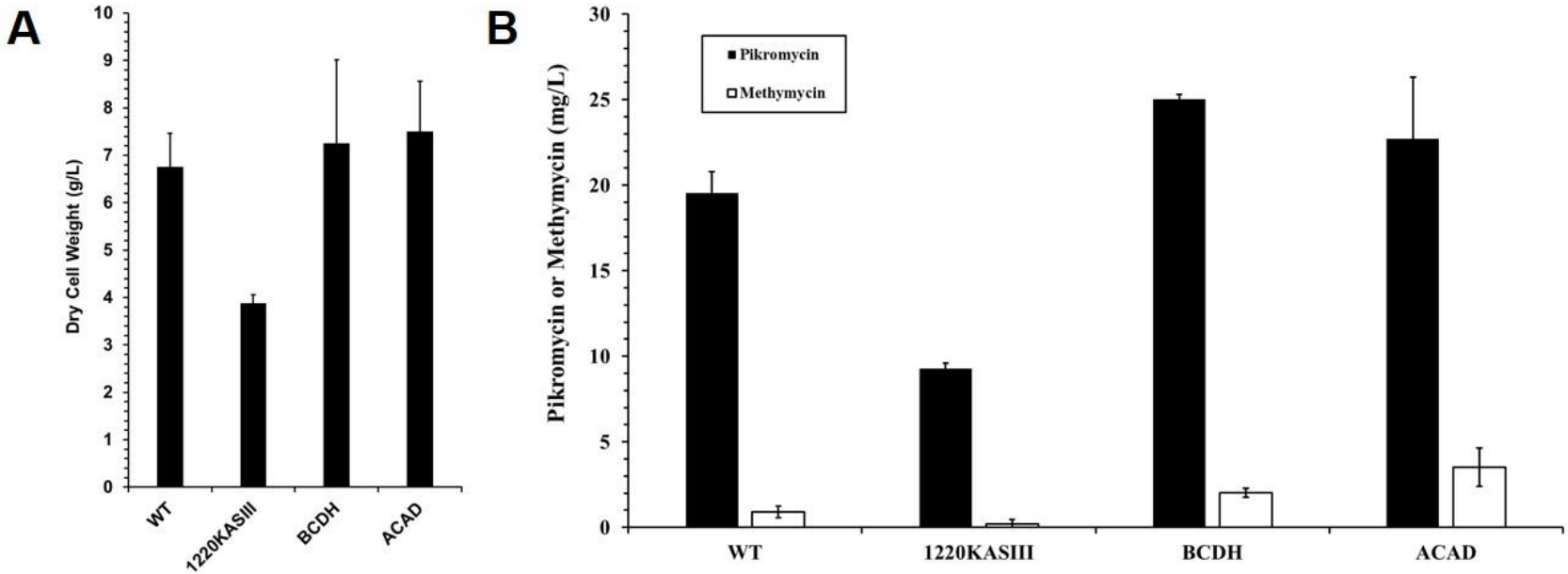
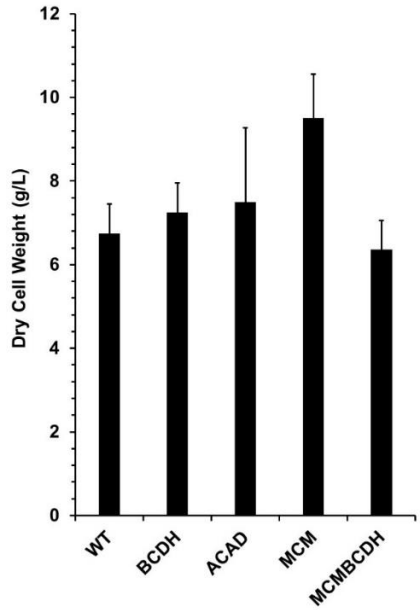


Figure 4.4. (a) DCW, and (b) pikromycin and methymycin productions from WT, KASIII repression mutant, and BCDH and ACAD overexpression mutants.

4.4.3 Double overexpression mutant of BCDH and MCM

A double overexpression mutant of BCDH and MCM ($SVEN_{mutA::bkd}$) was constructed to examine if there were any additive effects of the two genes on PKM production. From central carbon metabolism, methylmalonyl-CoA is produced from succinyl-CoA of TCA cycle by MCM. It is a well-known overexpression target, which increased methylmalonyl-CoA pool, and overproduced erythromycin by 1.63 folds from *Saccharopolyspora erythraea* (Reeves et al., 2007). MCM was a good overexpression candidate, along with BCDH, to maximize PKM production in *S. venezuelae* for that reason. All the individual enzymes were overexpressed with ermE promoter in pIBR25 plasmids. All the strains except $SVEN_{mutA}$ yielded almost the same DCW, about 7 g/L (Figure 4.5a). The cell mass of $SVEN_{mutA}$ was much higher than that of others, 9.5 g/L, suggesting that the contribution of the supply of methylmalonyl-CoA via TCA cycle possibly be more significant than that via BCAA degradation. The most PKM was produced from $SVEN_{mutA}$, 33.9 mg/L. Surprisingly, PKM titer of $SVEN_{mutA::bkd}$ decreased down to 25.0 mg/L (Figure 4.5b), which was almost the same level as that of $SVEN_{bkd}$. To further examine a reason for such decreases, we performed more detailed HPLC and LC-MS analysis. To further examine a reason for such decreases, we performed more detailed HPLC and LC-MS analysis. Three new compound peaks were detected from chromatograms of HPLC (Figure 4.6), and four from TSQ-MS analysis (Figure 4.7). The three newly detected compounds from HPLC were collected and further analyzed by MS/MS.

A



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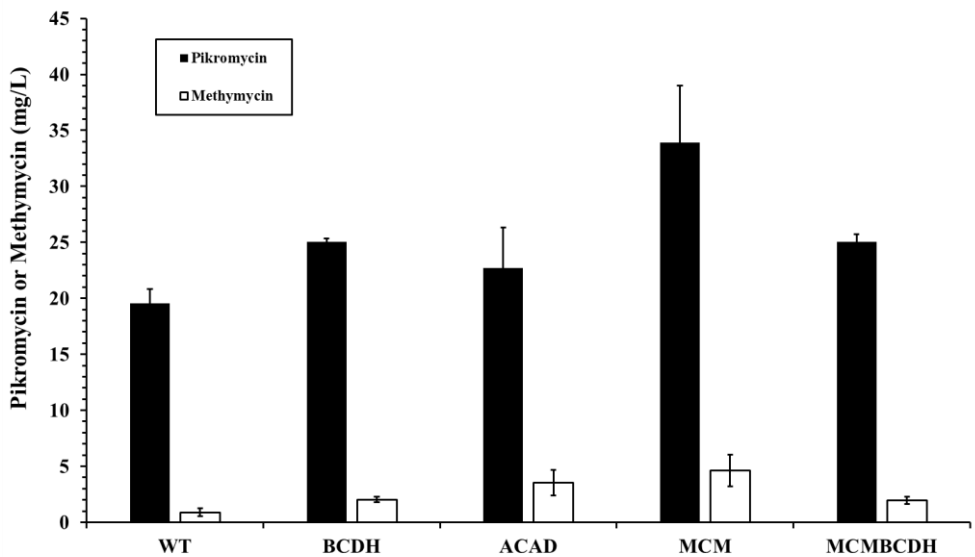


Figure 4.5. DCW of BCDH, ACAD, MCM and MCM+BCDH overexpression mutants.

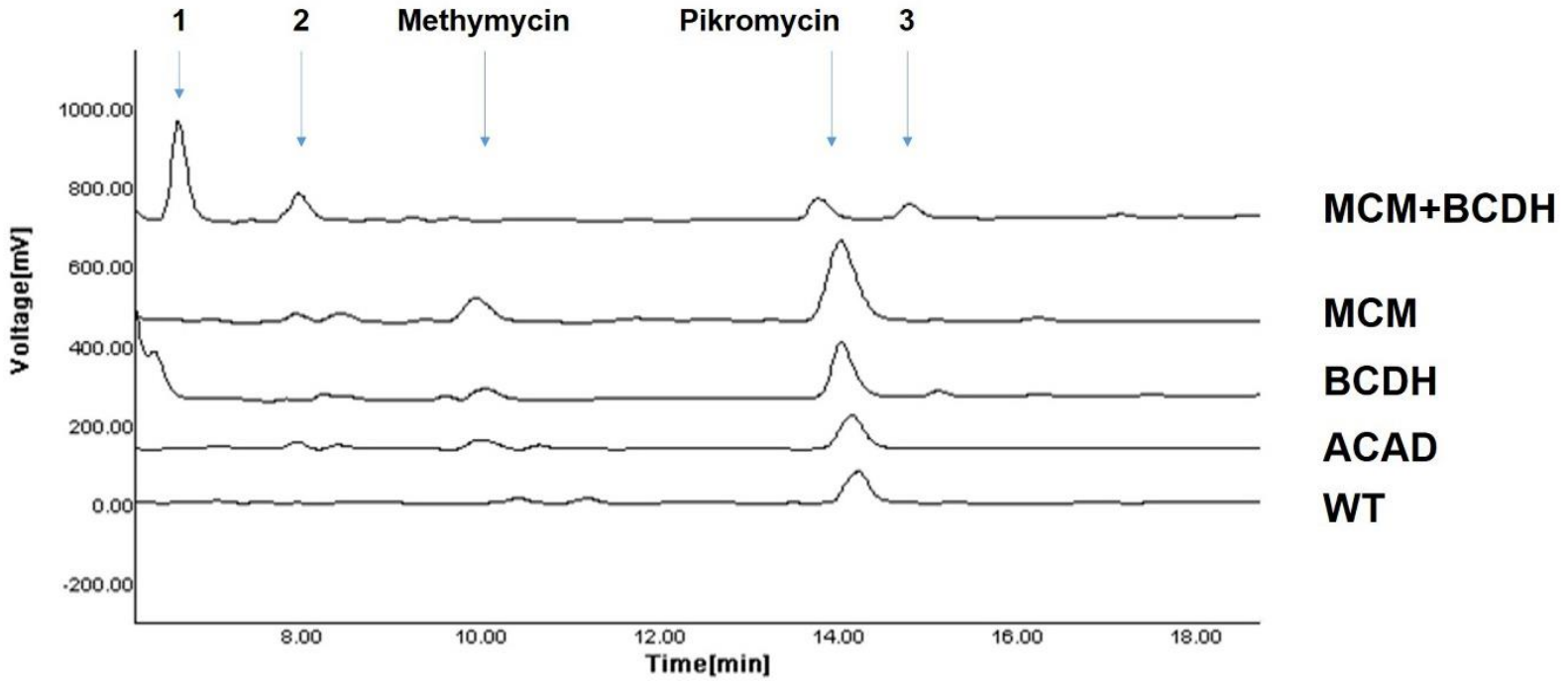


Figure 4.6. HPLC chromatograms from supernatant extracts of the WT, ACAD, BCDH, MCM, and BCDH + MCM overexpression mutants. Previously unidentified peaks at (1) 6.1 min, (2) 7.4 min, and (3) 14.5 min are detected from $SVEN_{bkd::mutA}$. They were further analyzed with TSQ-MS/MS.

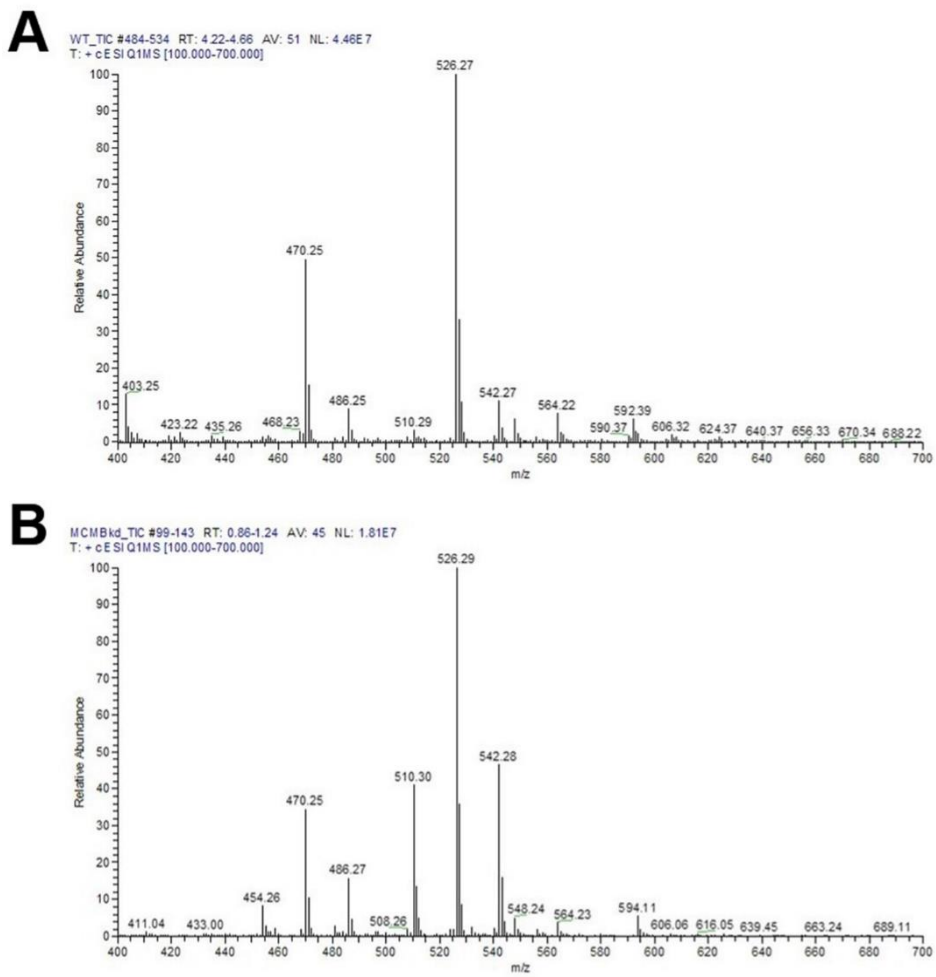
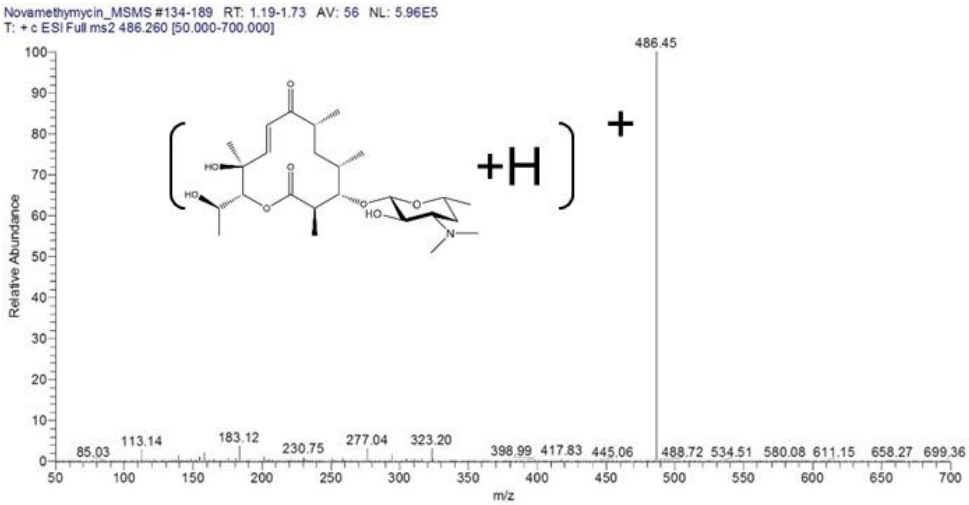


Figure 4.7. TSQ-MS total ion chromatogram of extracts from (a) WT and (b) BCDH + MCM double overexpression mutant. About the same amounts of narbomycin and novapikromycin with methymycin were measured from the double overexpression mutant using TSQ-MS analysis. Fair amounts of 10-deoxymethymycin and novamethymycin were also detected.

According to MS/MS analysis of the three unidentified compounds from $SVEN_{bkd::mutA}$ culture extracts, the three peaks were 486.45 m/z, 470.40 m/z, and 510.53 m/z, identified as [Novamethymycin + H]$^+$ (Figure 4.8a), [Neomethymycin + H]$^+$ (Figure 4.8b), and [Narbomycin + H]$^+$ (Figure 4.9a) respectively. Novapikromycin was also detected from crude extract of PK antibiotics from $SVEN_{bkd::mutA}$ (Figure 4.9b). Nova-forms are C12 hydroxylated forms of MTM and PKM. In $SVEN_{bkd::mutA}$, fair amounts of deoxy forms of PK antibiotics were accumulated, which may explain the decreases in the PKM production.

A



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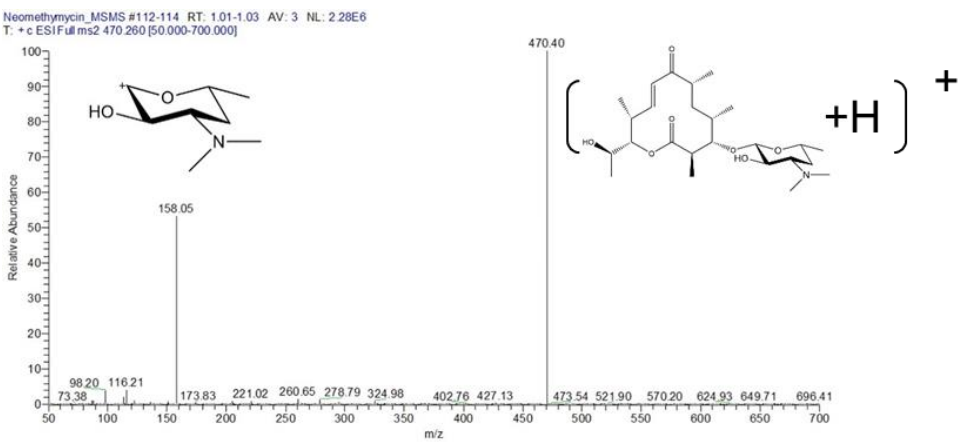

Figure 4.8. MSMS fragmentation of (a) novamethymycin and (b) neomethymycin. Mother peak of novamethymycin is at [novamethymycin+H] $^{+}$, 486.45 m/z. The mother peak of neomethymycin was at [neomethymycin+H] $^{+}$, 470.40 m/z, and [desosamine-OH]$^{+}$ was at 158.06 m/z.

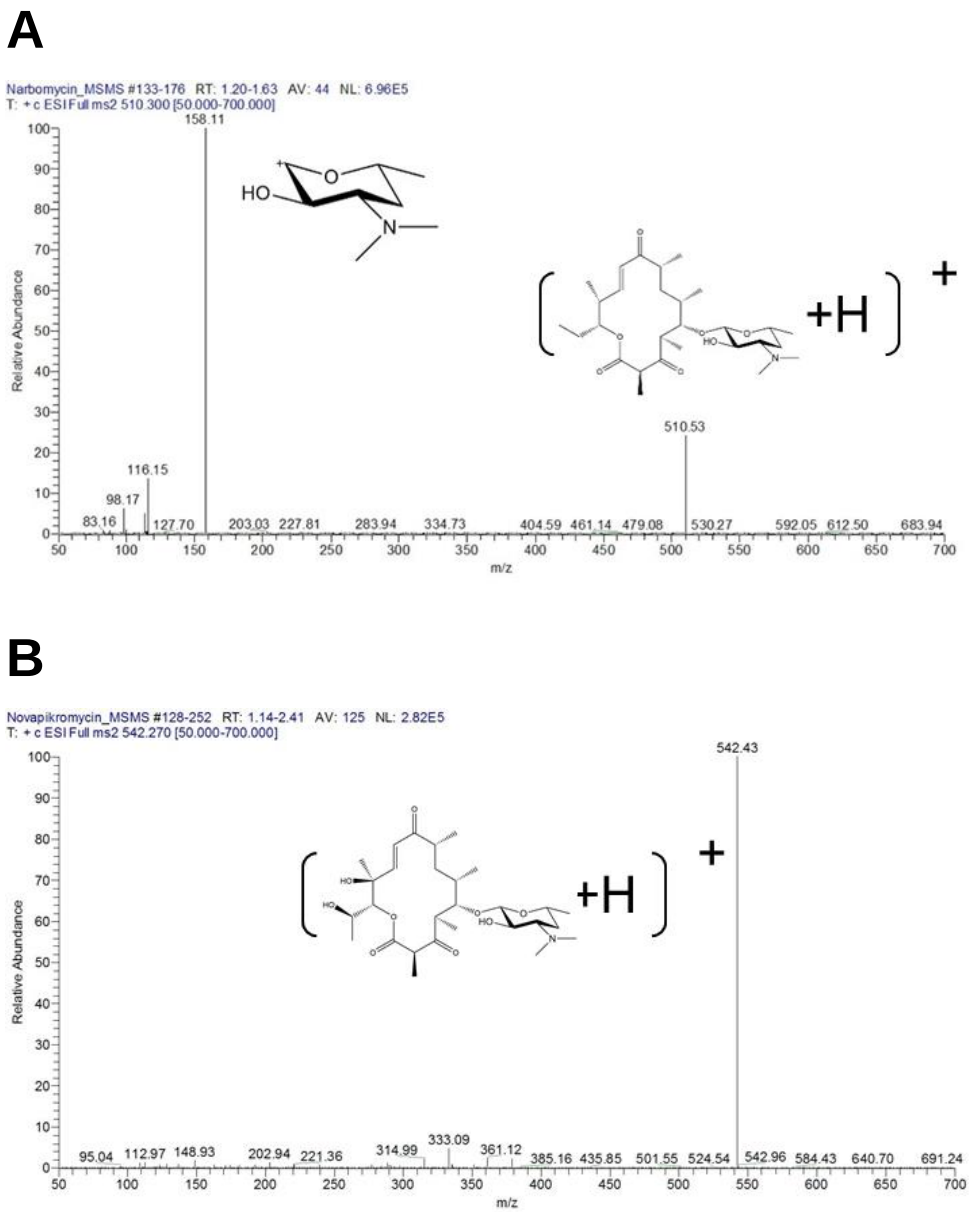

Figure 4.9. MSMS fragmentation of (a) narbomycin and (b) novapikromycin. Mother peak of narbomycin was at [narbomycin+H] $^{+}$, 510.53 m/z, and [desosamine-OH]$^{+}$ is at 158.11 m/z. Mother peak of novapikromycin is at [novapikromycin+H] $^{+}$, 486.45 m/z.

Despite the reduction of PKM production in $SVEN_{mutA::bkd}$ compared to that of $SVEN_{mutA}$, total macrolide productions increased on the other hand (Figure 4.10). Total 43 mg/L of macrolides were produced from $SVEN_{mutA::bkd}$, which was about 2 fold increases compared to that of the WT. It was 3.54 mg/L more macrolides compared to that of $SVEN_{mutA}$. The decrease of PKM, and surge of other macrolides in $SVEN_{mutA::bkd}$ strain indicated that supplementation of acyl-CoA precursors from metabolism was no longer a bottleneck within $SVEN_{mutA::bkd}$. But activities of *pikC*, cytochrome P450 monooxygenase, became a new bottleneck.

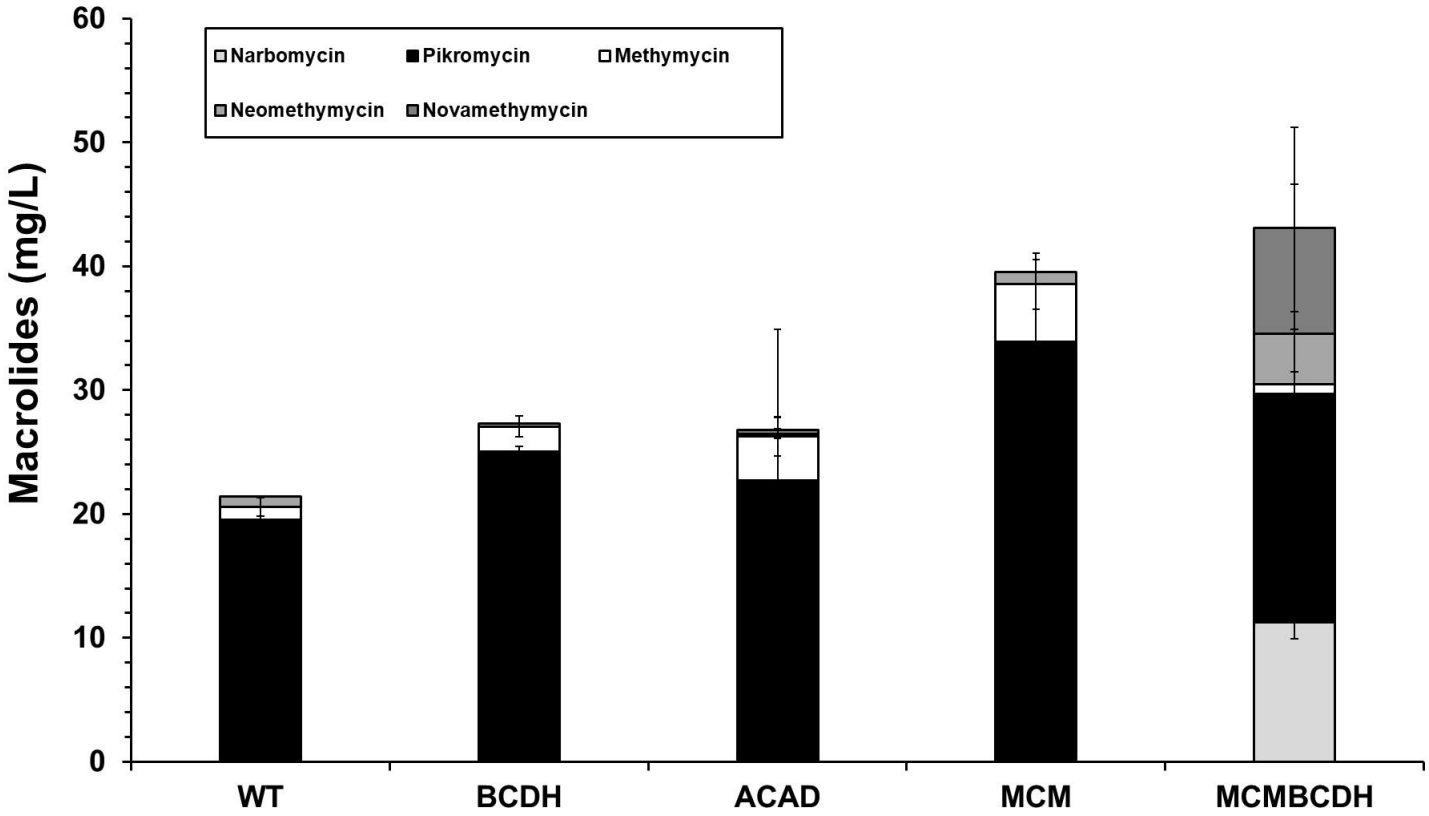

Figure 4.10. Macrolides productions from WT, SVEN$_{bkd}$, SVEN$_{acdH}$, SVEN$_{mutA}$, and SVEN$_{mutA::bkd}$.

In order to convert the accumulated deoxy-form antibiotics into MTM and PKM, it would be necessary to overexpress *pikC*, which is liable for the hydroxylation of most of PK metabolites produced by *S. venezuelae*. The enzyme appears to have somewhat broad substrate specificity. For PKM family, *pikC* recognizes desosamine groups, and adds hydroxyl groups at C12 and C14 positions (Srinivasan *et al*., 2004; Lee *et al*., 2006). *pikC* alone produces three different PKM and three different MTM derivatives (Figure 4.11). For that reason, *pikC* overexpression would result in not only hydroxylation of narbomycin and 10-deoxymethymycin, but also oxidation of PKM and MTM into their nova-forms. But to overcome the problem of intermediate accumulations, overexpression of *PikC* in $SVEN_{bkd::mutA}$ was attempted.

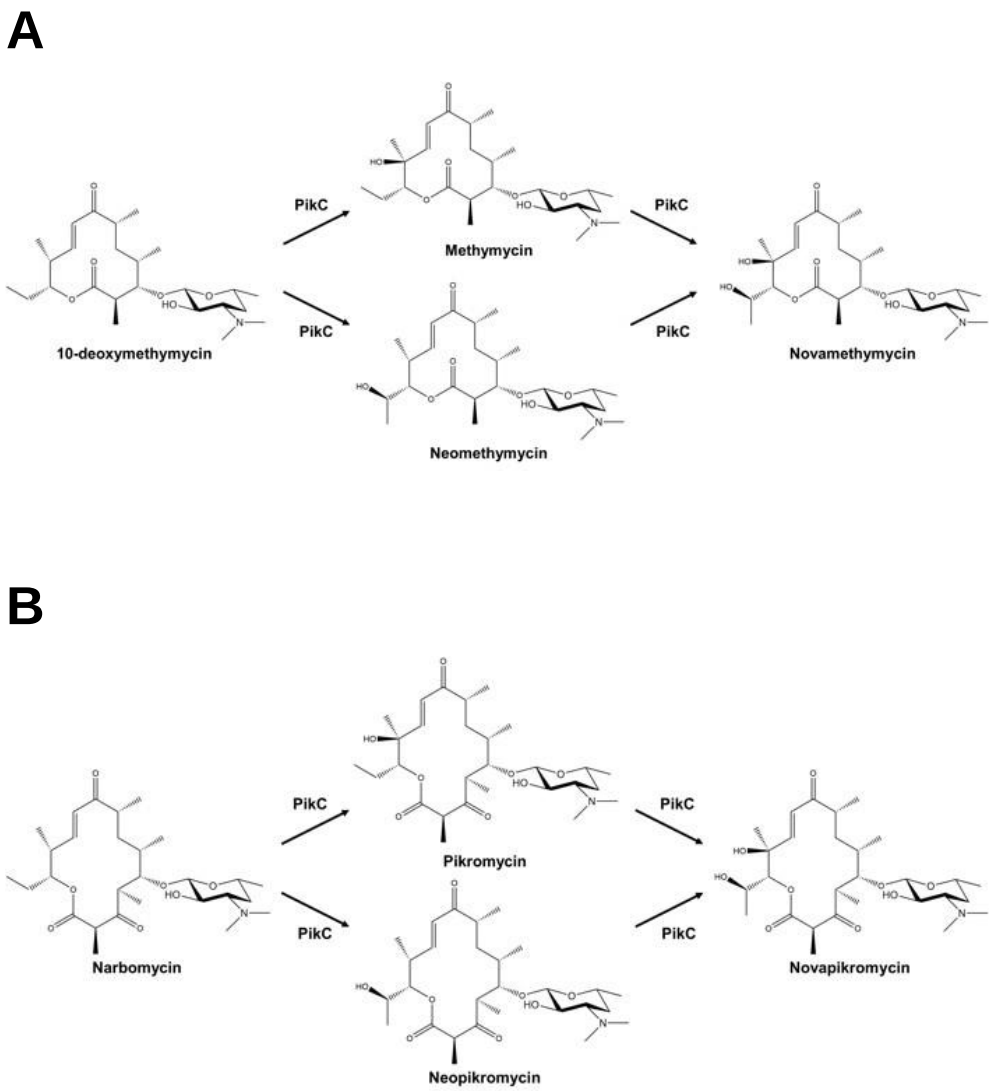

Figure 4.11. *pikC*, P450 mono-oxygenase, hydroxylation of (a) methymycin, and (b) pikromycin derivatives. A broad substrate specificity results in hydroxylation.

To make a stable expression of *pikC*, the gene was integrated into the chromosome ($SVEN_{pikC}$). Interestingly enough, transformation of pIBR25BM into $SVEN_{pikC}$ was not possible, because the transformed cells died (data not shown). There are some reports mentioning that P450 overexpression in *E. coli* reduces cell growth fitness (Julsing *et al*., 2008), but there were no vivid differences in growth rates of the WT and $SVEN_{pikC}$. However, whenever *pikC* was overexpressed with other enzymes, via either integrated into chromosome or plasmid amplification, *S. venezuelae* cells died, which we do not understand well yet. As a result, we are suggesting that there needs another method to hydroxylate narbomycin and 10-deoxymethymycin.

In result, conversion of narbomycin into PKM was not possible with *pikC* overexpression, but a possible strategy to solve such problem would be modifications of *pikC* substrate specificity. As long as desosamine was attached to polyketide compounds, *pikC* was shown to hydroxylate many of them in the previous reports, resulting in numerous hydroxylated aminoglycoside products (Li *et al*., 2009). Taking advantages of its large substrate specificity, *pikC* was mutated to accept various substrates with different amino sugars or types of aglycones (Negretti *et al*. 2014). If *pikC* could be mutated to accept narbomycin and nothing else, it would be possible to further maximize the PKM production.

4.5 Conclusion

Streptomyces venezuelae, producing mainly PKM and MTM, received spotlights as a good heterologous expression host due to its fast growing nature compared to other *Streptomyces* sp. (Phelan *et al*., 2017). In this study, synthesis of methylmalonyl-

CoA from BCAA degradation was applied in *S. venezuelae* by manipulating expressions of three key enzymes involved. In *S. coelicolor*, it was previously reported that BCAA catabolism showed similar contributions towards acetyl-CoA for ACT production with carbon metabolism, and its importance in polyketide antibiotics was emphasized (Stirrett *et al*., 2009; Kim *et al*., 2014). Unfortunately, however, methylmalonyl-CoA coming from succinyl-CoA of TCA cycle had greater influence than that from BCAA degradation towards PKM productions in *S. venezuelae*. The highest titer of PKM was achieved by MCM overexpression, resulting in 1.7 fold increases, 33.9 mg/L, compared to that of the WT. Overexpression of BCDH and MCM together resulted in the highest macrolide production titer of 43 mg/L. But intermediates and other forms of PKM and MTM began to be accumulated, suggesting enzymatic activities or control nods of *pikC* (i.e. gene regulation) were altered. It would be necessary to manipulated activities or substrate specificities of *pikC* to accept only narbomycin as a substrate in BCDH and MCM double overexpression mutant to further maximize PKM productions.

Chapter 5.

Overall Conclusion and Further Suggestions

5.1 Production of fatty acids and polyketides from acyl-CoA metabolism

Despite the fact that several thousands of secondary metabolites are produced from Streptomyces species, there are still a lot more to find out (i.e. regulatory networks of biosynthetic pathways, cofactor regenerations, and cryptic genes that are expressed in certain conditions). Developments of synthetic tools, such as next generation sequencing (NGS) and clustered regularly interspaced short palindromic repeats (CRISPR), allowed detailed analysis and engineering of *Streptomyces* genome. In this study, synthetic tools for gene expressions in *Streptomyces coelicolor* was first developed based on multi-omics datasets from NGS. Total eight sets of promoter / 5' UTRs were constructed and successfully applied to express genes at various strengths.

Next, branched chain amino acid (BCAA) metabolism was utilized to produce branched chain fatty acids (BCFA) and pikromycin (PKM). Since BCFA and PKM uses the same acyl-CoA precursor pools, the same engineering strategies, i.e. push, pull, and redirection, could be applied to maximize their productions. Overexpression of branched-chain alpha-keto acid dehydrogenase (BCDH) pushed BCAA catabolism, and overexpression of 3-ketoacyl acyl carrier protein synthase III (KASIII) or acyl-CoA dehydrogenase (ACAD) each pulled acyl-CoA fluxes towards fatty acid synthesis or methylmalonyl-CoA synthesis respectively. It was successful to increase BCFA productions using BCAA catabolism, but for PKM, it seemed that carbon metabolism would supply more acyl-CoA precursors than

BCAA metabolism. Taken together, this thesis may provide general guide line for genome engineering to produce various type of polyketide secondary metabolites or BCFAs with branching points at desired C positions.

5.2 Further suggestion

Rewiring metabolism and pathway optimization are performed to maximize productions of valuable compounds. Such optimizations are performed by multiplex automated genome engineering (MAGE) or MAGE coupled to CRISPR (CRMAGE). They are oligonucleotide mediated genome engineering techniques (Wang et al., 2009), which means they require DNA editing templates. Promoter and 5' UTR gene expression sets developed in *S. coelicolor* could be used as a template for pathway optimization by MAGE or CRMAGE. For example, in case of PKM, glucose is required for synthesis of both, desosamine and narbonolide. Desosamine is synthesized from glucose-1-phosphate through 6 enzymatic steps, while narbonolide is produced from methylmalonyl-CoA, which is produced from glycolysis and tricarboxylic acid (TCA) cycle (Wilson et al., 2001) (Figure 5.1). MAGE could be applied to balance the carbon flux and optimize PKM productions by finding out optimum expression levels of genes participating in each steps of PKM biosynthesis.

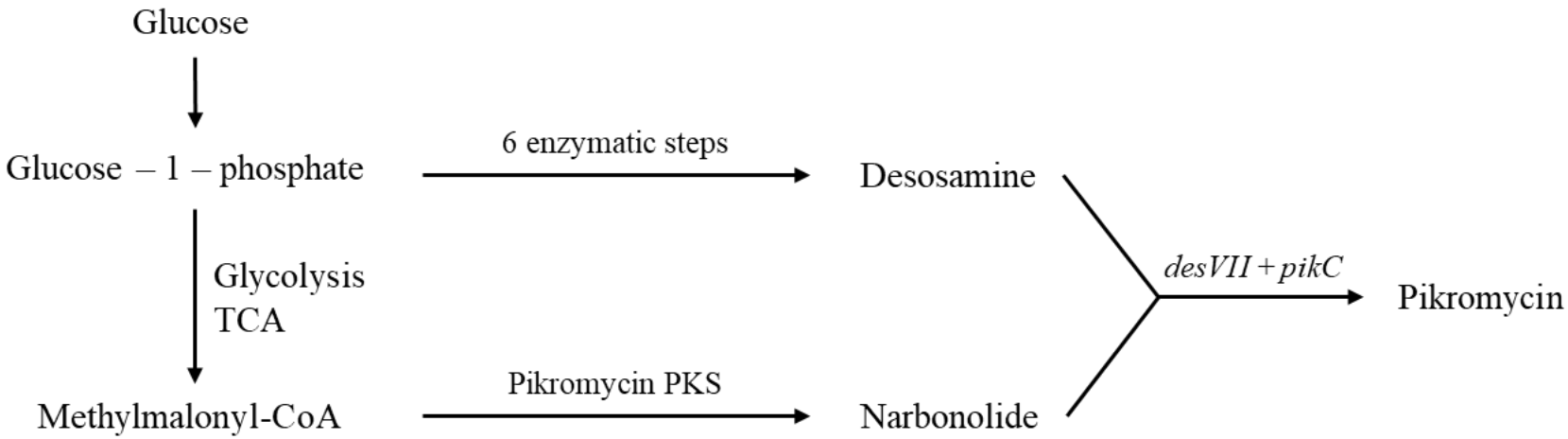

Figure 5.1. Desosamine and narbonolide production pathway for pikromycin biosynthesis.

Cofactor biosynthesis and regenerations are another important factors in biosynthesis of fatty acids and polyketide antibiotics. Reductases in polyketide sythase (PKS) and fatty acid synthase (FAS) genes utilize NADPH for reduction of keto groups during chain elongation process (Sanli and Blaber, 2001). However, intracellular percentages of NADH is higher than that of NADPH (Chun et al., 2007; Ying, 2008). For that reason, NADPH balancing and regenerations have been keys to cofactor studies (Sung et al., 2015). In this thesis, only the effects of C and N source catabolism were examined. And the overexpression of BCDH was a key factor in BCAA catabolism. If BCDH was collaborated with NAD(P)H transhydrogenase, *pntAB*, it may provide a functional cofactor regeneration system for PKS and FAS genes (Figure 5.2). BCDH uses NAD^+, and generates NADH in the process of BCAA catabolism (Denoya et al., 1995). Because *pntAB* produces NADPH from NADH, it may act as a simple cofactor regeneration system, bridging precursor generations and its chain elongations. It may ultimately result in an optimized production host for secondary metabolite productions.

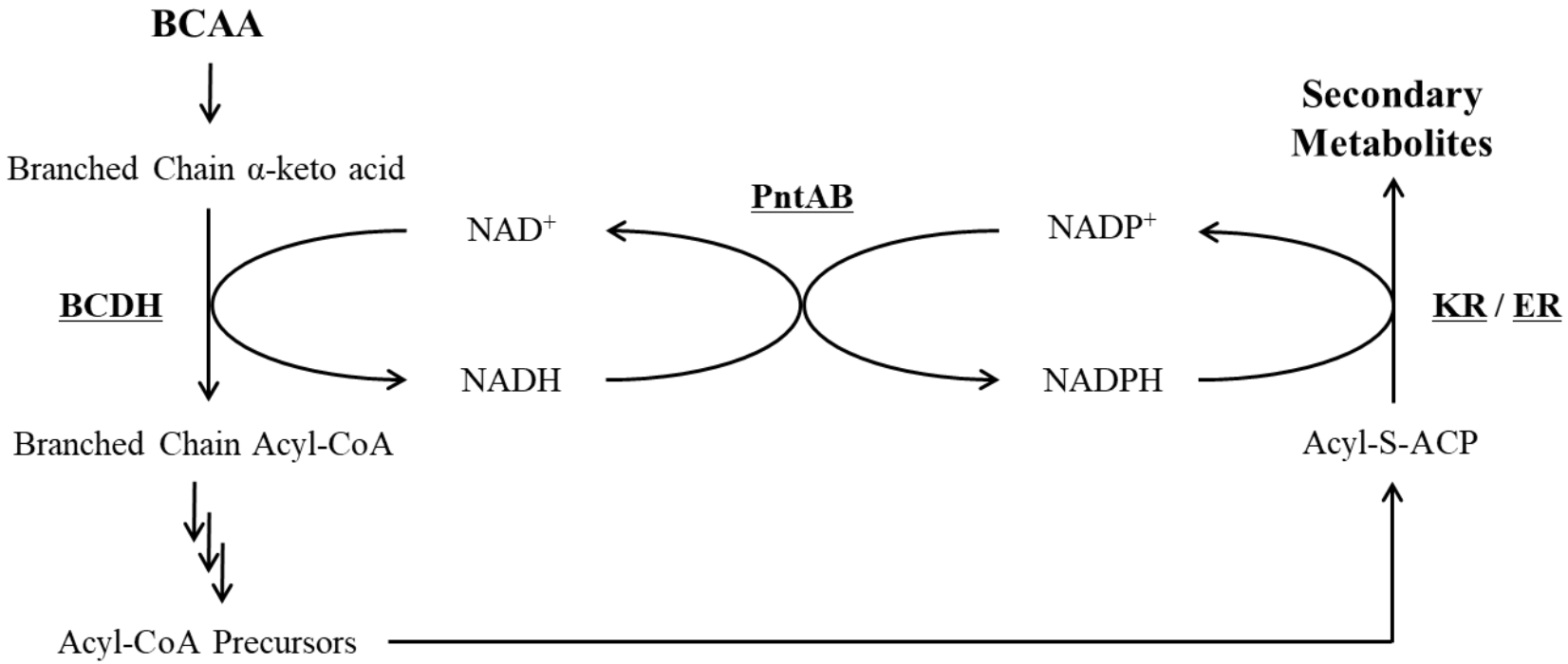

Figure 5.2. Cofactor regeneration system using BCDH and NAD(P)H transhydrogenase.

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Appendix

Effects of Sucrose, Phosphate, and Calcium Carbonate on the Productions of Pikromycin from *Streptomyces venezuelae* ATCC 15439

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A.1 Abstract

Polyketide secondary metabolites share common precursor pools, acyl-CoA. Thus effects of engineering strategies for heterologous and native secondary metabolite productions are often determined by measurement of pikromycin in *Streptomyces venezuelae*. It is hard to compare effectiveness engineering targets among published data due to different pikromycin production media used one from the other. To determine the most important nutritional factor and establish optimal culture conditions, media optimization of pikromycin from *Streptomyces venezuelae* ATCC 15439 was studied with a statistical method, Plackett-Burman design. 9 variables, glucose, sucrose, peptone, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $CaCO_3$ were analyzed for their effects on a response, pikromycin. Glucose, K_2HPO_4, and $CaCO_3$ were determined to be the most significant factors. Path of the steepest ascent and response surface methodology about the three selected components were performed to study interactions between the three factors, and fine-tune concentrations for maximized product yields. The significant variables and optimal concentrations were 139 g/L sucrose, 5.29 g/L K_2HPO_4, and 0.081 g/L $CaCO_3$, with the maximal pikromycin yield of 35.5 mg/L. Increases of the antibiotics production by 1.45-fold, 1.3-fold, and 1.98-fold, compared with unoptimized medium, and other two pikromycin production media SCM and SGGP respectably, were achieved.

Keywords: *Streptomyces venezuelae*, Pikromycin, Media optimization, Response Surface Methodology

A.2 Introduction

Large numbers of secondary metabolites such as antibiotics and anti-tumor drugs are produced by microorganisms, and about 7600 are known to be produced by *Streptomyces* species (Berdy, 2005). Fast and non-aggregating growth characteristics that allow large cell mass, and efficient profiling of primary and secondary metabolites give attentions to *Streptomyces venezuelae*, producing mainly pikromycin and methymycin, be used as a good heterologous expression host (Thapa et al., 2007). Pikromycin is a polyketide secondary metabolite, a 14-membered cyclized macrolactone narbonolide with a desosamine that has hydroxylation at a specific carbon (Maezawa et al., 1973). Due to the nature of polyketide sharing common acyl-CoA precursors, results of engineering strategies for enhancing the heterologous or native secondary metabolite productions are often screened by measurement of antibiotics pikromycin (Xue et al., 1998; Maharjan et al., 2008).

Productions of secondary metabolites with *Streptomyces venezuelae* are often conducted in many different media. The strain is inoculated from fermentation media to three different production media for pikromycin alone (Chen et al., 2000; Chen et al., 2001; Lee et al., 2006), which result in altered metabolic behaviors leading to changes in titers of product yields. Since the titers and growth conditions are different from one another, it gives a complication with research conductions and data comparisons, such as microarray and ChIP data, as a result. Because gene manipulations of *Streptomyces* species are difficult and limited compared to that of

other microorganisms like *Escherichia coli* (Burke et al., 2001), resolving this complication is crucial for selecting a right engineering target for host generations.

The aim of this research was to determine the most significant factors and create an optimized medium for productions of pikromycin in *Streptomyces venezuelae*, and increase the yield at the same time. In order to do so, Plackett-Burman design and statistical method, response surface methodology, was applied to identify and fine tune the concentrations of significant media components.

A.3 Materials and Methods

A.3.1 Bacterial strain and culture conditions

Streptomyces venezuelae ATCC 15439, pikromycin and methymycin producing wild type, was used for the media optimization study. Chemicals were purchased from BD (San Jose, CA, USA) and Junsei (Tokyo, Japan). Liquid R2YE complex media (10 g glucose, 103 g sucrose, 5 g yeast extract, 0.1 g Difco Casamino Acids, 0.25 g K_2SO_4, 10.12 g $MgCl_2 \cdot 6H_2O$, 2 mL of a trace element solution composed of 200 mg $FeCl_3 \cdot 6H_2O$, 40 mg $ZnCl_2$, 10 mg $MnCl_2 \cdot 4H_2O$, 10 mg $CuCl_2 \cdot 2H_2O$, 10 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 mg $Na_2B_4O_7 \cdot 10H_2O$, 10 mL of 0.5% KH_2PO_4, 4 mL of 5 M $CaCl_2 \cdot 2H_2O$, 15 mL of 20% L-proline in 1 liter of distilled water, 5.73 g TES [*N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]* buffer, and 7 mL of 1 N NaOH in 1 liter of distilled water) was used for propagation of the strain. Productions of pikromycin was performed in non-optimized medium (20g glucose, 103g sucrose,

10g peptone, 2.5g $(NH_4)_2SO_4$, 2.5g K_2HPO_4, 2.5g KH_2PO_4, 1g NaCl, 1g $MgSO_4 \cdot 7H_2O$, 0.2g $CaCO_3$ in 1 liter of distilled water).

Strain was seed cultured at 100 mL of R2YE, 30℃ with shaking at 200 rpm for 18 hours. 200 mg wet weighted cells were inoculated in 50 mL non-optimized medium, also grown at 30℃ and 200 rpm shaking, for 60 hours. The maximum production time point was different from one media to another, due to different nutritional sources and concentrations. The main culture period was determined from preliminary experiments with a common pikromycin production medium, SCM. No significant increase in pikromycin production was observed after 60 hours of the culture, thus that time point was used throughout the experiments for comparison of pikromycin productions from different media. (Figure A.1)

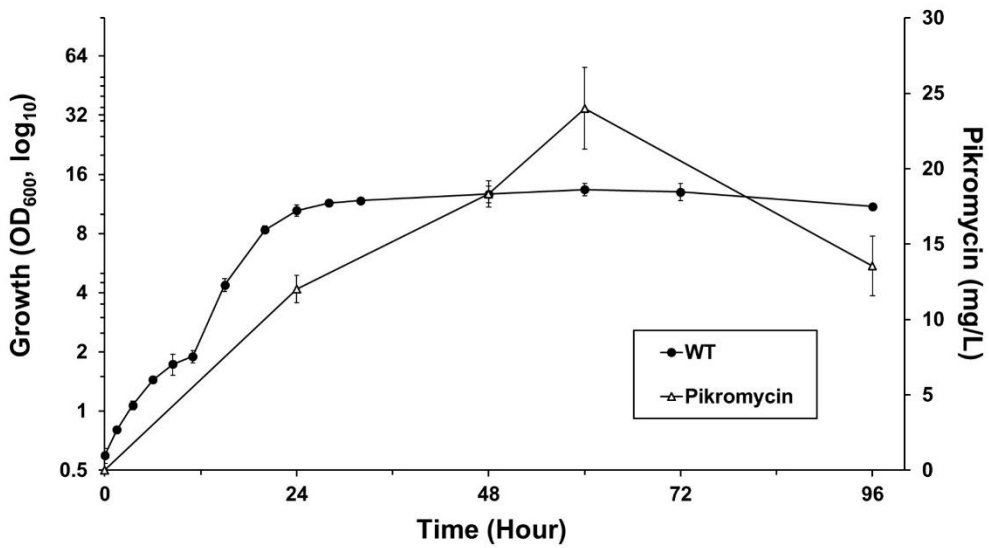


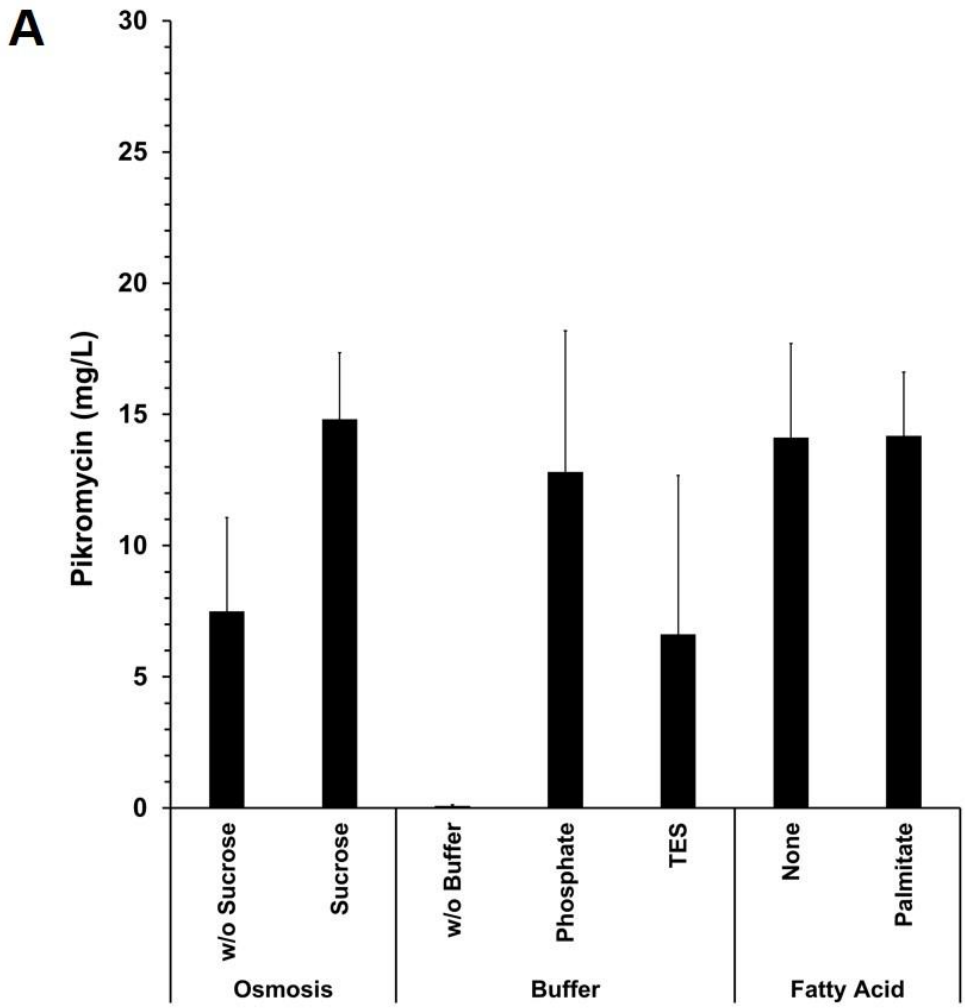
Figure A.1. **Growth of *S. venezuelae* WT and corresponding pikromycin productions.** *S. venezuelae* enters a stationary phase on day 1 of the culture. Maximum pikromycin production was observed around day 3 of the culture, and it decreased dramatically afterwards.

A.3.2 Quantification of pikromycin productions

15 mL of the cultures was harvest by centrifugation at 2840 x g for 10 min. Pikromycin was extracted from supernatants with 2 volume of ethyl acetate. The extract was dried using a rotary vacuum evaporator and reconstituted with 0.68 mL methanol. 20 μL of the samples were analyzed by high-performance liquid chromatography (HPLC, YL-9100, Younglin, Korea). Reverse phase C_{18} column from Waters (Milford, MA, USA) was used with a linear gradient from solvent A (80:20 water / 80% acetonitrile in 5 mM ammonium acetate and 0.05% acetic acid) to solvent B (20:80 water / 80% acetonitrile in 5 mM ammonium acetate and 0.05% acetic acid) over 25 min at a flow rate of 1 mL/min. Detection was made at 220 nm, and pikromycin corresponding peak was confirmed by LCQ-LC/MS.

A.3.3 Experimental design and data analysis

From preliminary studies testing various C and N sources, osmotic stress, buffers, and fatty acids, one media component from each sources that resulted in the most pikromycin production was selected. (Figure A.2) Glucose, sucrose, peptone, ammonium sulfate, monobasic and dibasic phosphate, sodium chloride, magnesium sulfate, and calcium carbonate were selected for further optimizations.


A
Pikromycin (mg/L)
30
25
20
15
10
5
0
w/o Sucrose
Sucrose
w/o Buffer
Phosphate
TES
None
Palmitate
Osmosis
Buffer
Fatty Acid

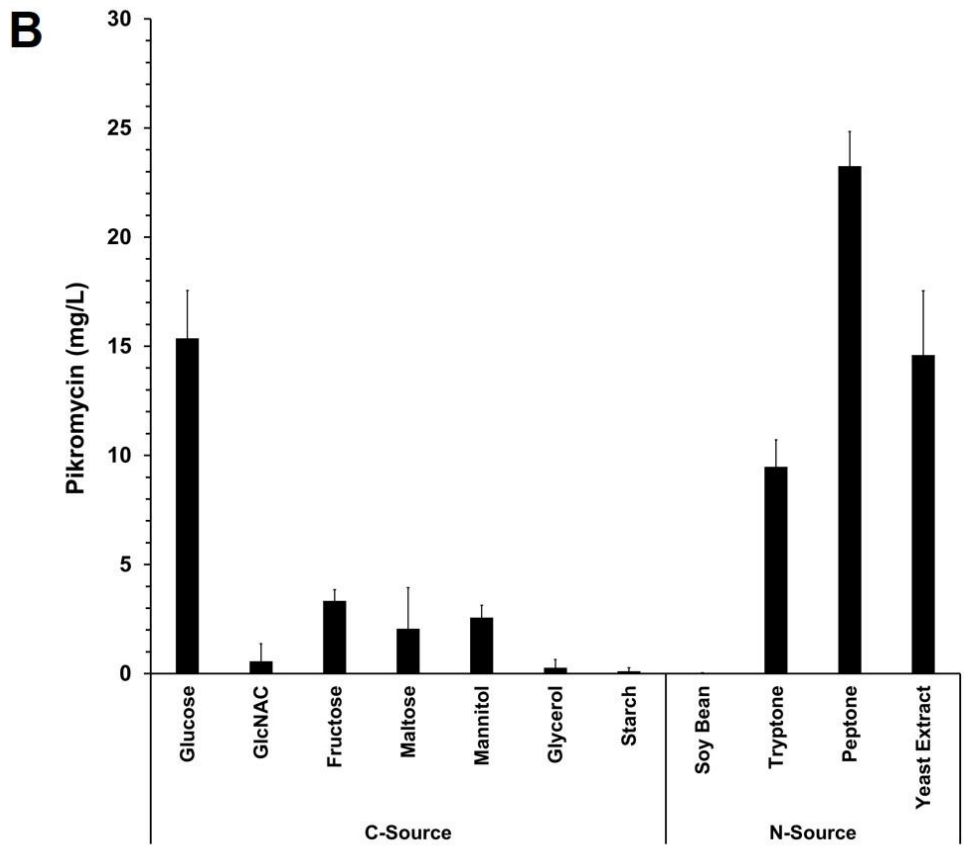


Figure A.2. Effects of various nutrients on pikromycin productions. (a) Production of pikromycin versus sucrose, phosphate and TES buffers, and palmitic acid. (b) Production of pikromycin versus various C and N sources.

Minitab 14.12 (Minitab Inc., Pensylvania, USA) was used to design and analyze data throughout the experiments. To identify the most significant variables in pikromycin production, Plackett-Burman Design (PBD) was employed. 11 variables, nine components and two dummies, were included in the design of total 12 experimental sets. (Table A.1) Two dummy variables, which had no chemical meanings to the production of pikromycin, were included to calculate random measurement errors. The random measurement errors were used to determine the significance of real values by calculating *F* values of *F*-test from Minitab software.

PBD results were reinterpreted to study correlations between pikromycin productions and cell mass. With the same PBD matrix design (Table A.2), significance of the factors were observe with cell mass as response.

Table A.1. PBD matrix design, real values of each coded variables, and responses, pikromycin

	Variables											Pikromycin
Runs	A	B	C	D	E	F	G	H	I	J[a]	K[a]	(mg/L)
1	1	1	-1	1	-1	-1	-1	1	1	1	-1	16.3
2	1	1	1	-1	1	1	-1	1	-1	-1	-1	30.6
3	-1	1	-1	-1	-1	1	1	1	-1	1	1	15.7
4	1	1	-1	1	1	-1	1	-1	-1	-1	1	30.3
5	1	-1	-1	-1	1	1	1	-1	1	1	-1	5.6
6	-1	1	1	-1	1	-1	-1	-1	1	1	1	17.3
7	-1	-1	1	1	1	-1	1	1	-1	1	-1	28.5
8	-1	-1	-1	1	1	1	-1	1	1	-1	1	1.5
9	1	-1	1	-1	-1	-1	1	1	1	-1	1	9.8
10	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1.0
11	1	-1	1	1	-1	1	-1	-1	-1	1	1	1.2
12	-1	1	1	1	-1	1	1	-1	1	-1	-1	13.7

a. Dummy factors. Nothing was added for the two to study errors.

Codes	Glucose	Sucrose	Peptone	$(NH_4)_2SO_4$	K_2HPO_4	KH_2PO_4	NaCl	$MgSO_4 \cdot 7H_2O$	$CaCO_3$
(g/L)	A	B	C	D	E	F	G	H	I
-1	14	72.1	7	2	1.8	1.8	0.7	0.7	0.14
1	26	134	13	3	3.3	3.3	1.3	1.3	0.26

Table A.2. PBD matrix design, real values of each coded variables, and responses, cell mass

	Variables											Cell Mass
Runs	A	B	C	D	E	F	G	H	I	J	K	(g/L)
1	1	1	-1	1	-1	-1	-1	1	1	1	-1	28.0
2	1	1	1	-1	1	1	-1	1	-1	-1	-1	22.4
3	-1	1	-1	-1	-1	1	1	1	-1	1	1	29.8
4	1	1	-1	1	1	-1	1	-1	-1	-1	1	30.5
5	1	-1	-1	-1	1	1	1	-1	1	1	-1	34.9
6	-1	1	1	-1	1	-1	-1	-1	1	1	1	29.0
7	-1	-1	1	1	1	-1	1	1	-1	1	-1	33.8
8	-1	-1	-1	1	1	1	-1	1	1	-1	1	34.6
9	1	-1	1	-1	-1	-1	1	1	1	-1	1	33.4
10	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	19.6
11	1	-1	1	1	-1	1	-1	-1	-1	1	1	32.1
12	-1	1	1	1	-1	1	1	-1	1	-1	-1	31.4

Codes	Glucose	Sucrose	Peptone	$(NH_4)_2SO_4$	K_2HPO_4	KH_2PO_4	NaCl	$MgSO_4 \cdot 7H_2O$	$CaCO_3$
(g/L)	A	B	C	D	E	F	G	H	I
-1	14	72.1	7	2	1.8	1.8	0.7	0.7	0.14
1	26	134	13	3	3.3	3.3	1.3	1.3	0.26

Path of the steepest ascent was performed to setup basal concentrations of media components selected from PBD to be used in Central Composite Design (CCD). Path of the steepest ascent allowed rapid movement towards the optimum of variable concentrations. The amounts were altered by increasing or decreasing according to the result of PBD. The combination of variable concentrations that resulted in maximum production indicated that it was at the point near optimum (Gheshlaghi et al., 2005).

Response Surface Methodology (RSM), one of CCD methods, was performed to find the optimal concentrations of variables determined to be important from PBD to maximize the productions of pikromycin. Matrix design and levels of components are listed in Table A.3.

Table A.3. RSM matrix design, real values of variables, and the results

Runs	Variables			Pikromycin (mg/L)
	Sucrose	K_2HPO_4	$CaCO_3$	
1	1.633	0	0	24.4
2	0	0	-1.633	28.4
3	0	-1.633	0	37.0
4	0	1.633	0	26.1
5	0	0	1.633	30.4
6	0	0	0	30.4
7	0	0	0	25.8
8	-1.633	0	0	25.1
9	0	0	0	25.4
10	-1	1	-1	25.0
11	-1	-1	1	32.5
12	1	1	1	22.9
13	0	0	0	25.7
14	1	-1	-1	26.8
15	0	0	0	24.0
16	0	0	0	26.4
17	-1	-1	-1	25.6
18	-1	1	1	21.9
19	1	-1	1	26.0
20	1	1	-1	20.5

Factors (g/L)	Codes	-1.633	-1	0	1	1.633
Sucrose	*X1*	104.0	123.6	154.5	185.4	205.0
K_2HPO_4	*X2*	5.3	6.0	7.0	8.1	8.7
$CaCO_3$	*X3*	0.049	0.055	0.065	0.075	0.081

A.4 Results and Discussion

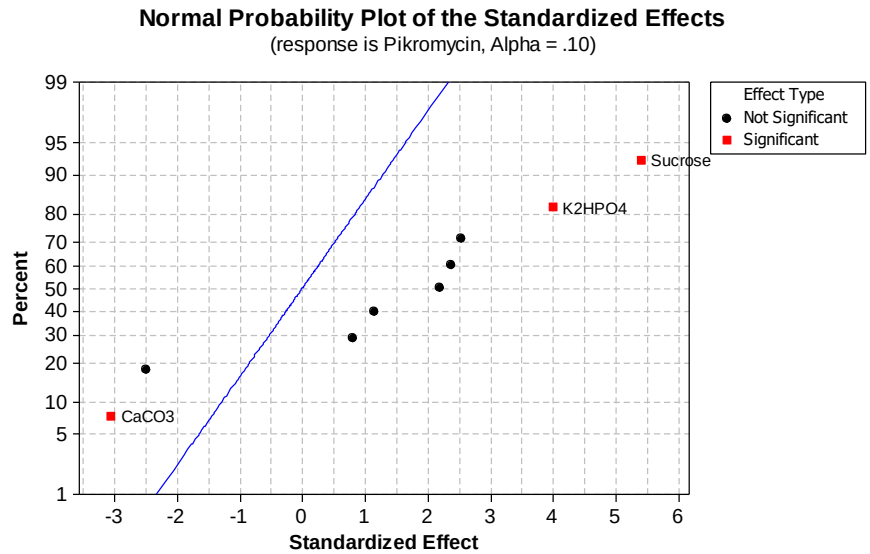
A.4.1 Identification of the most significant variables with PBD

Various stress conditions applied during growth of *Streptomyces* species are known to alter secondary metabolites productions. It has been reported that secondary metabolism of *Streptomyces coelicolor* begins during pre-sporulating stage of cell cycle (Yague et al., 2013), which means that the time point of the secondary metabolite production can be actively altered by availability of nutrients. Nutrient sources may also change the metabolism that different carbon and nitrogen sources result in various titers (Yu et al., 2008). PBD experiments tested the importance of the nine factors listed in the Table A.4.

The result showed effects of variables to the response, pikromycin. Effects of sucrose, K_2HPO_4, and $CaCO_3$ were (+) 6.346, (+) 4.686, and (-) 3.578 respectably, which meant that sucrose and K_2HPO_4 had positive, and $CaCO_3$ had negative effects to the response. The three components were also determined to be the most significant in pikromycin productions, based on the low *P*-values (<0.1). R^2 was found to be 0.9754, meaning that 97.54% of the total variations could be explained by the model. On the Pareto chart of the standardized effects (Figure A.3), the minimal effects were presented towards lower fields, near 0, and the maximal effects towards upper fields.

Table A.4. Effects of variables to the response, pikromycin

Factors	Effect	Coefficient	T	P
Glucose	2.678	1.339	1.14	0.372
Sucrose	12.692	6.346	5.41	0.033
Peptone	5.122	2.561	2.18	0.161
$(NH_4)_2SO_4$	1.905	0.953	0.81	0.502
K_2HPO_4	9.372	4.686	3.99	0.057
KH_2PO_4	-5.825	-2.912	-2.48	0.131
NaCl	5.942	2.971	2.53	0.127
$MgSO_4 \cdot 7H_2O$	5.562	2.781	2.37	0.141
$CaCO_3$	-7.155	-3.578	-3.05	0.093
R^2 = 97.54%	R^2 (adj) = 86.48%			



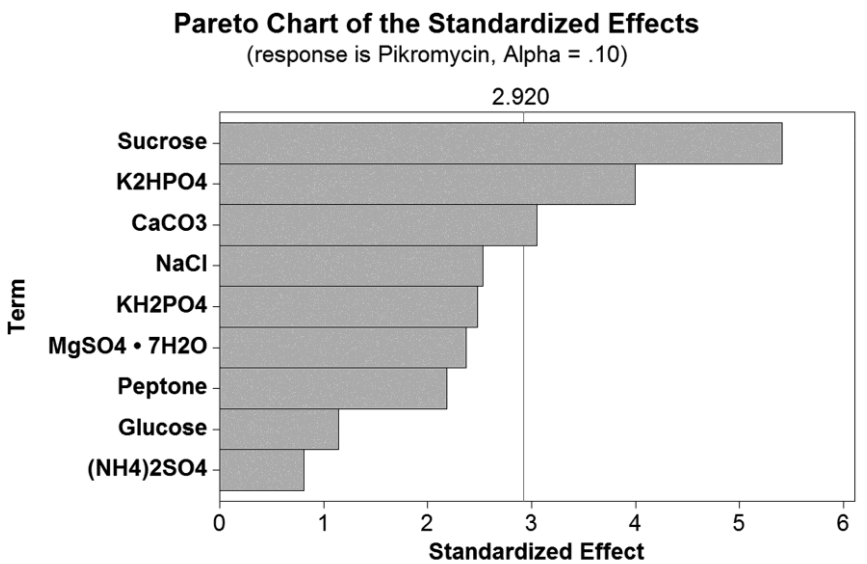


Figure A.3. Pareto chart of the standard effects of the tested nine factors to pikromycin productions. Sucrose, K_2HPO_4, and $CaCO_3$ were determined to be significant while C and N sources were not.

An essential element for microorganisms, which applies the same for all living organisms, is phosphorus. Formation of a fuel compound ATP (Rodríguez-García et al., 2009), DNA and RNA synthesis (Prokofieva-Belgovskaya and Popova, 1959), and many other biochemical process occurring in living creature require phosphorus (Hošťálek et al., 1976). A primary source of phosphorus in bacterial cultures is K_2HPO_4, and KH_2PO_4. K_2HPO_4 was determined to be more suitable source of phosphorus to produce pikromycin from the results of PBD. It is expected that a precursor of desosamine from glycone portion of pikromycin, glucose-1-phosphate (Xue and Sherman, 2001), can better be synthesized from K_2HPO_4. Alternatively, optimization of pH by altering a ratio of monobasic and dibasic phosphate (Green, 1933) may have dedication to the increase of the pikromycin productions as reported in pH sensitive production of actinorhodin with *Streptomyces coelicolor* (Bystrykh et al., 1996; Kim et al., 2007).

Just as phosphorus, sucrose is an important carbon source for plants and some phytopathogenic bacteria (Bogs and Geider, 2000). However it is used somewhat differently in cultures of *Streptomyces* species. Sucrose cannot be utilized with the species (Reid and Abratt, 2005) that it is often used as one of compounds reducing osmotic stress, one of key components in the secondry metabolism (Pettis and Cohen, 1996; Huang et al., 2001; Takano et al., 2003). A previous report indicated an absence of sucrose banished productions of antibiotics actinorhodin in *Streptomyces coelicolor*, and an optimization of sucrose for production media has been further studied (Elibol and Mavituna, 1998).

Calcium ions are often used for increases of enzymatic stabilities (Spungin and Blumberg, 1989; Chakraborty et al., 2009). But calcium is known to decrease secondary metabolite productions. From the past studies, increases in calcium ions supplement resulted in larger total mass of *Streptomyces coelicolor*, but the opposite was observed with the productions of actinorhodin (Abbas and Edwards, 1990). Secondary metabolism shares common precursor and cofactor pools like acetyl-CoA and NADPH (Drew and Demain, 1977; Komatsu et al., 2010), that calcium may have contributions to balancing fluxes of the compounds toward growth and secondary metabolism, and the result of PBD in this study indicate. $CaCO_3$ is determined to have negative effects on pikromycin productions while positive effects on cell mass. In other words, some loss in cell mass should be endured for gain in pikromycin productions. Importance of the nine factors (Table A.5), when the response was cell mass, indicated that $CaCO_3$ were found to be positively significant, but sucrose was negatively significant towards cell mass (Figure A.4) which was totally opposite from that of when pikromycin was the response. Thus the concentrations of calcium carbonate was minimized to a satisfactory level with RSM.

Table A.5. Effects of variables to the response, cell mass

Factors	Effect	Coefficient	T	P
Glucose	0.521	0.26	0.18	0.875
Sucrose	-2.896	-1.448	-0.99	0.428
Peptone	0.771	0.385	0.26	0.817
$(NH_4)_2SO_4$	3.562	1.781	1.21	0.348
K_2HPO_4	1.813	0.906	0.62	0.6
KH_2PO_4	1.812	0.906	0.62	0.6
NaCl	4.646	2.323	1.58	0.254
$MgSO_4 \cdot 7H_2O$	0.729	0.365	0.25	0.827
$CaCO_3$	3.854	1.927	1.31	0.319

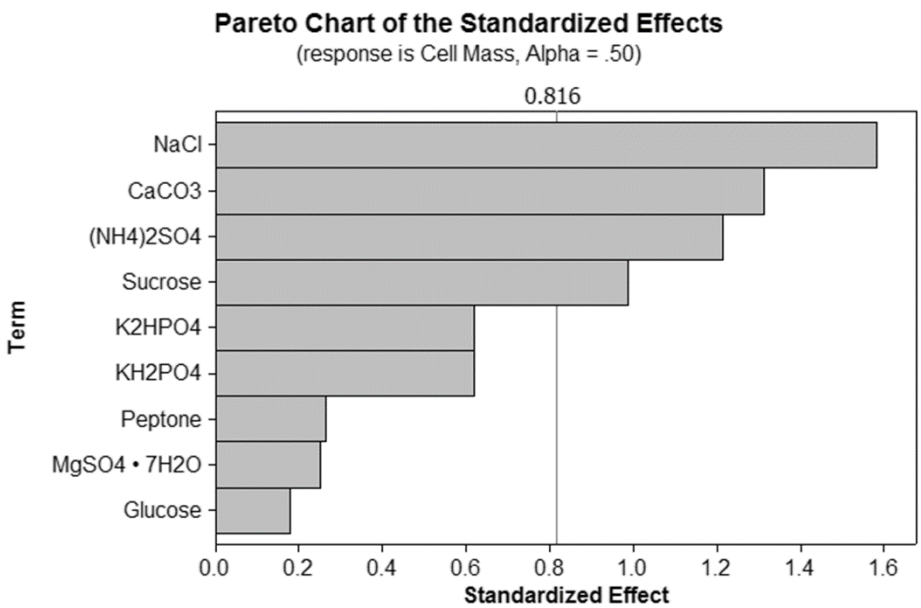


Figure A.4. Pareto chart of the Standardized Effects with cell mass as a response.

NaCl, $CaCO_3$ and $(NH_4)_2SO_4$ were found to be positively, while sucrose was negatively significant towards cell mass.

A.4.2 Path of the steepest ascent

Concentrations of sucrose and K_2HPO_4 were increased, but that of $CaCO_3$ was decreased because $CaCO_3$ was determined to have negative effects on pikromycin productions (Table A.6). All the other components were fixed at the concentrations of the non-optimized medium. When the concentrations of sucrose, K_2HPO_4, and $CaCO_3$ were 154.5 g/L, 3.75 g/L, and 0.1 g/L respectably, production of pikromycin was maximal, 28.5 mg/L. This point was chosen as a clue to set up a basal concentrations for further optimizations.

Table A.6. Design of path of the steepest ascent, and the results

	Variables			Pikromycin
Runs	Sucrose (g/L)	K_2HPO_4 (g/L)	$CaCO_3$ (g/L)	(mg/L)
1	103	2.5	0.2	19.3
2	133.9	3.25	0.14	23.8
3	154.5	3.75	0.1	28.5
4	175.1	4.25	0.06	26.3
5	195.7	4.75	0.02	25.1
6	206	5	0	19.0

A.4.3 Response Surface Methodology

Specific interactions between the three variables, response to pikromycin productions, were studied with RSM (Figure A.5). Effects of the three components, sucrose, K_2HPO_4, and $CaCO_3$ were analyzed by *t*-Test and *P*-values as shown in table 5. A regression model with R^2 higher than 0.9 meant that the result had high correlation between predicted and experimental values of the response (Chen et al., 2009). And it also meant that the model could explain 91.2% of the total variations. This model can be expressed as the equation (A.1).

$$Y = 26.2912 - 0.7551\ X_1 - 2.9028\ X_2 + 0.6478\ X_3 - 1.4306\ X_1{*}X_1 + 1.1231\ X_2{*}X_2 + 0.3112\ X_3{*}X_3 + 0.2138\ X_1{*}X_2 - 0.2737\ X_1{*}X_3 - 0.8513\ X_2{*}X_3 \quad \text{(A.1)}$$

Analysis of variance (ANOVA), Table A.7, also provided reliability of the model from the statistically significant regression ($P < 0.01$), and the statistically insignificant lack of fit ($P = 0.778$). As a result, the model was determined to be reliable and adequate to optimize the production of pikromycin.

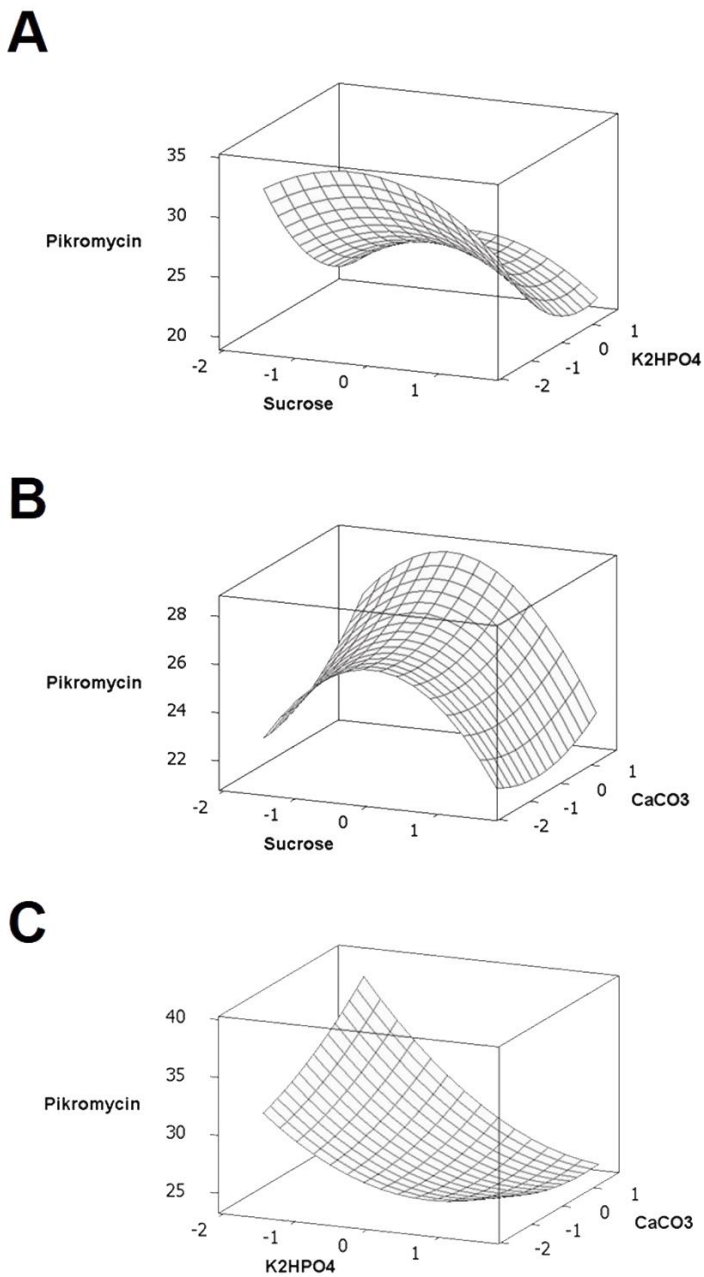


Figure A.5. 3D surface graph of SRM. (a) sucrose vs. K_2HPO_4, (b) sucrose vs. $CaCO_3$, and (c) K_2HPO_4 vs. $CaCO_3$ for the response, pikromycin.

Table A.7. ANOVA test of the regression model

Source	DF [a]	Seq SS [b]	Adj SS [b]	Adj MS [c]	F	P
Regression	9	180.736	180.736	20.082	6.75	0.007
Linear	3	125.547	125.547	41.849	14.07	0.001
Square	3	48.426	48.426	16.142	5.43	0.025
Interaction	3	6.762	6.762	2.254	0.76	0.548
Residual Error	8	23.792	23.792	2.974		
Lack-of-Fit	5	10.591	10.591	2.118	0.48	0.778
Pure Error	3	13.201	13.201	4.4		
Total	19	270.181				

a. DF = Degree of Freedom

b. SS = Sum of Squares

c. MS = Mean Square

A.4.4 Validation of the optimized medium

The full quadratic model from RSM predicted that the maximum production of pikromycin was 38.6 mg/L at 139 g/L sucrose, 5.29 g/L K_2HPO_4, and 0.081 g/L $CaCO_3$. Validation experiments with the optimized condition resulted in 35.5 ± 0.866 mg/L of pikromycin, which were in near agreement. The yield from unoptimized medium was 24.4 mg/L that 1.45-fold increase was achieved. 1.98-fold and 1.29-fold increase in pikromycin productions also had been obtained, compared to the other media SGGP (17.9 ± 2.43 mg/L) and SCM (27.4 ± 1.05 mg/L) respectably. Production of pikromycin per gram cell from the optimized medium was 34.1 ± 0.852 mg/L per g cell, which was 4.19 folds and 3.2 folds higher than that of SGGP and SCM respectably.

A.5 Conclusion

Medium optimization of pikromycin production was achieved by the statistical designs of experiments in this study. First, PBD was applied to select the most significant factors. On the second step, CCD was used to fine tune the optimal concentrations of the selected variables. With the optimal concentrations at 139 g/L sucrose, 5.29 g/L K_2HPO_4, and 0.081 g/L $CaCO_3$, maximum pikromycin yields of 35.5 mg/L was achieved. 1.45 fold had been increased compared to non-optimized medium, and 1.98-fold and 1.29-fold increase were achieved compared to SGGP and SCM respectably. Thus our media may be employed to other studies related to pikromycin productions.

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국문 초록

본 연구는 다양한 가지사슬 acyl-CoA 의 생합성 경로를 이용하여 가지사슬 지방산 및 이차대사산물의 생산을 극대화 시킬 수 있도록 하였다. *Streptomyces venezuelae* 와 *Streptomyces coelicolor* 에서 각각 pikromycin (PKM) 항생제와 가지사슬 지방산 생산을 하기 위해 가지사슬 아미노산 이화작용을 이용하였다.

첫번째로 *S. coelicolor* 에서 가지사슬 지방산 생산을 하기위해 3-ketoacyl acyl carrier protein synthase III (KASIII)와 branched chain α-keto acid dehydrogenase (BCDH)을 actinothodin, undecylprodigiosin, a cryptic type I polyketide, calcium-dependent antibiotic 이 제거된 균주에서 과발현시켰다. 또한 베타 산화의 중요한 유전자인 *fadD* 를 제거하여 지방산이 분해되지 않도록 하였다.

S. venezuelae 에서 PKM 의 생산을 증가시키기 위해 가지사슬 아미노산 대사에 관여한 세가지의 효소, KASIII, acyl-CoA dehydrogenase (ACAD), BCDH 의 발현을 조절하였다. 합성 프로모터와 5' UTR 을 이용하여 KASIII 효소의 발현을 줄여 지방산 생산을 줄여보았으나, 성장이 저해되어 PKM 생산이 줄어드는 것을

확인 할 수 있었다. 따라서 BCDH 와 ACAD 를 과발현시켜 아미노산 대사를 이용하고자 하였고, 탄소 대사로부터 생산이 되는 것과 비교를 하였다. PKM 은 탄소 대사로부터 만드는 것이 더 효율적이라는 것을 밝혀냈으며, 탄소와 아미노산 대사를 동시에 증가시킬 경우, *pikC* 유전자의 활성이 속도 결정 단계가 되는 것으로 확인이 되었다.

본 연구는 가지사슬 아미노산의 대사공학 및 평가를 통하여 가지사슬 지방산과 pikromycin 항생제의 전구체인 acyl-CoA 생산을 증가시켰다. 이는 acyl-CoA 로 이루어진 다른 대사체 생산 연구 등에 통찰력을 줄 것으로 보여진다.

주요어: 방선균, 합성 프로모터, pikromycin, 가지사슬 아미노산, 가지사슬 지방산, polyketide synthase gene, fatty acid synthase genes, 베타산화

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