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FERMENTATION, CELL CULTURE AND BIOENGINEERING - ORIGINAL PAPER

Production of pikromycin using branched chain amino acid catabolism in *Streptomyces venezuelae* ATCC 15439

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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 *Streptomyces 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 increase 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 total macrolide production of 43 mg/L, which was about 2.2 fold increase compared to that of the WT. 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*

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 [18]. There are many efforts and studies to increase the productions of such secondary metabolites [11, 33] in academia as well as fermentation industry. Among them, flux balance between fatty acids (FA) and polyketide (PK) biosynthesis is one of general strategies to overproduce molecules of interests, because FAs and PKs share common precursor pools [1, 10]. 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 at their high level productions, due to similarities in their gene sequences and protein structures [4]. To increase PK antibiotics productions, the precursors in FA biosynthesis should be redirected towards PK biosynthesis.

Dynamic controlling fluxes 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 other antibiotics such as triclosan was newly suggested in *Streptomyces* sp. to achieve successful repression of fatty acids synthesis [33]. And, enhancements

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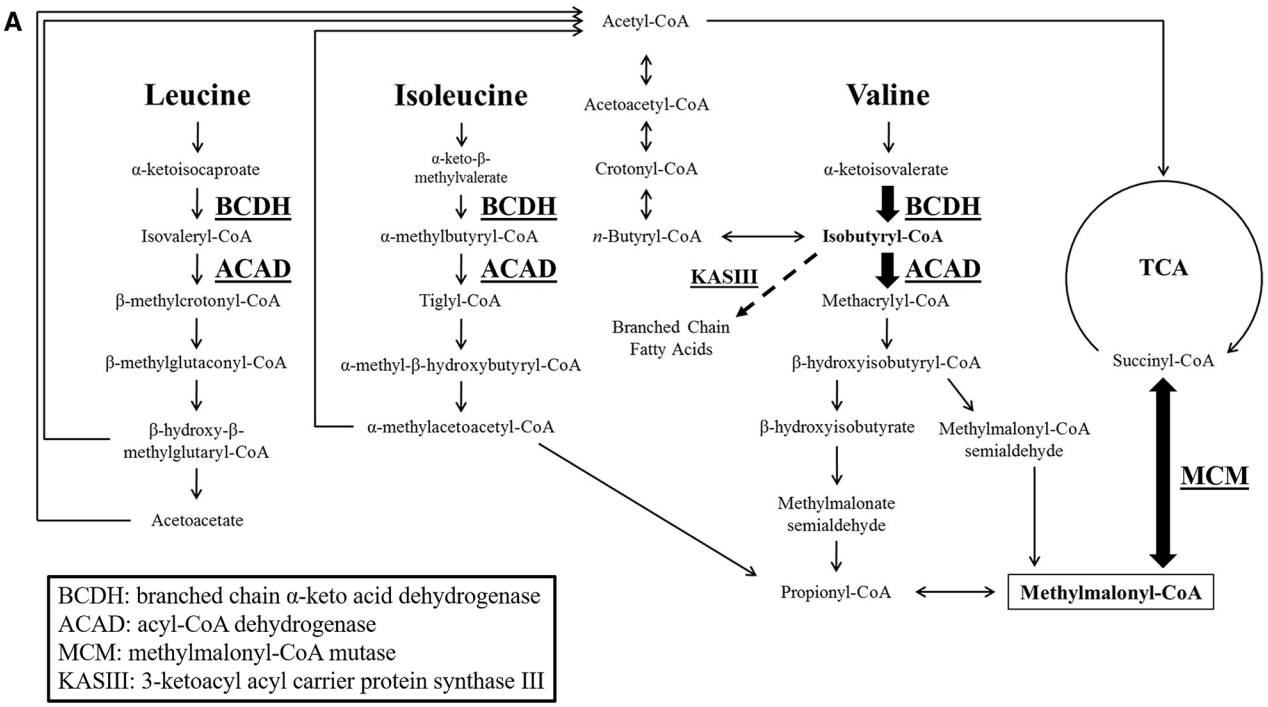
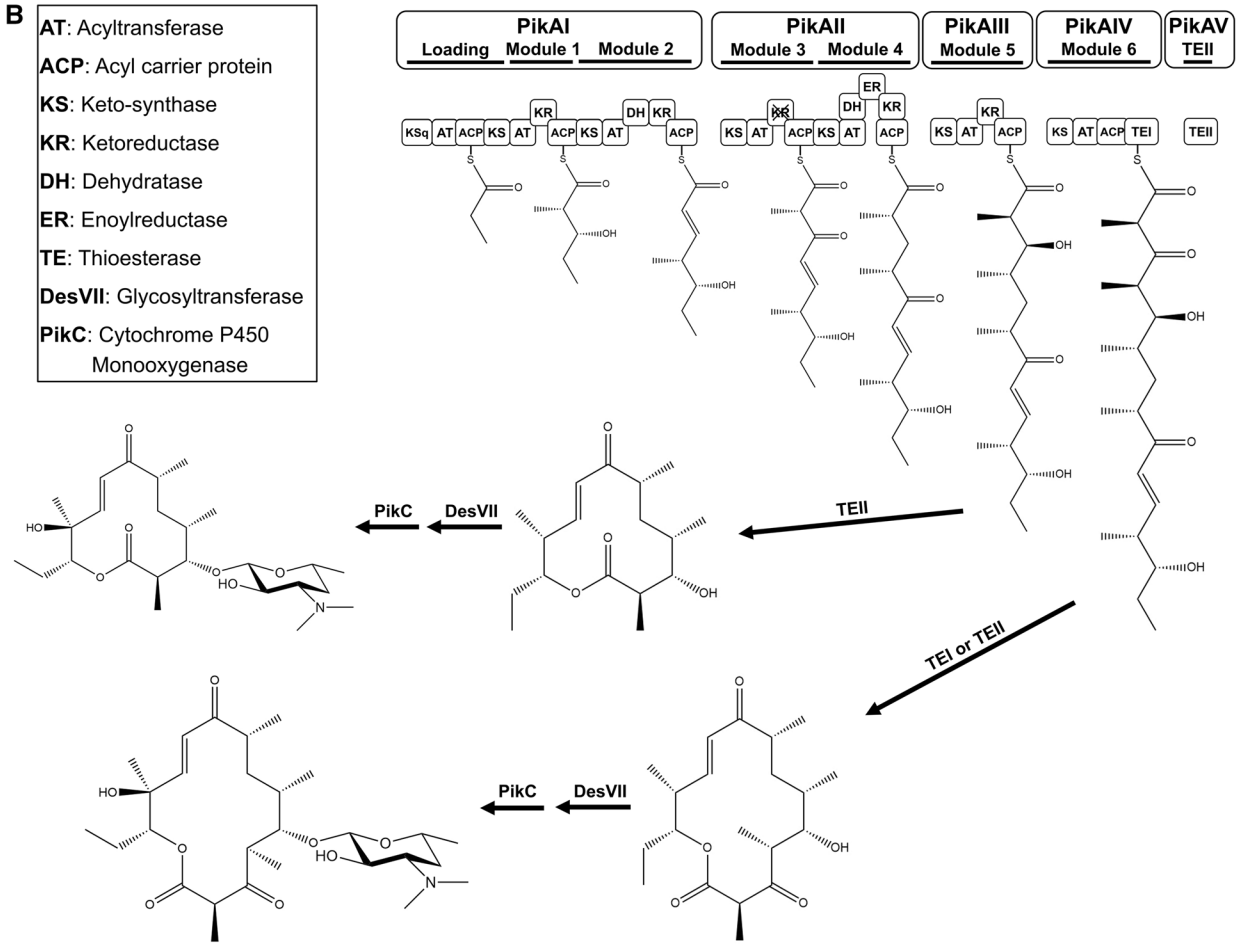

A
Acetyl-CoA
Leucine
α-ketoisocaproate
BCDH
Isovaleryl-CoA
ACAD
β-methylcrotonyl-CoA
β-methylglutaconyl-CoA
β-hydroxy-β-methylglutaryl-CoA
Acetoacetate
Isoleucine
α-keto-β-methylvalerate
BCDH
α-methylbutyryl-CoA
ACAD
Tiglyl-CoA
α-methyl-β-hydroxybutyryl-CoA
α-methylacetoacetyl-CoA
Acetoacetyl-CoA
Crotonyl-CoA
n-Butyryl-CoA
KASIII
Branched Chain Fatty Acids
Valine
α-ketoisovalerate
BCDH
Isobutyryl-CoA
ACAD
Methacrylyl-CoA
β-hydroxyisobutyryl-CoA
β-hydroxyisobutyrate
Methylmalonyl-CoA semialdehyde
Methylmalonate semialdehyde
Propionyl-CoA
Methylmalonyl-CoA
TCA
Succinyl-CoA
MCM
BCDH: branched chain α-keto acid dehydrogenase
ACAD: acyl-CoA dehydrogenase
MCM: methylmalonyl-CoA mutase
KASIII: 3-ketoacyl acyl carrier protein synthase III


B
AT: Acyltransferase
ACP: Acyl carrier protein
KS: Keto-synthase
KR: Ketoreductase
DH: Dehydratase
ER: Enoylreductase
TE: Thioesterase
DesVII: Glycosyltransferase
PikC: Cytochrome P450 Monooxygenase
PikAI
Loading
Module 1
Module 2
PikAII
Module 3
Module 4
PikAIII
Module 5
PikAIV
Module 6
PikAV
TEII
KSq
AT
ACP
KS
KR
DH
ER
TEI
TEII
PikC
DesVII
TEII
TEI or TEII

◂**Fig. 1** **a** TCA cycle and valine catabolism pathway leading to methylmalonyl-CoA. Overexpression targets are indicated by bold arrow, and repression target by dashed arrow. **b** Pikromycin (PKM) and methymycin (MTM) biosynthesis pathway. PKM and MTM are produced from type I PKS genes, composed of 6 modules, from PikAI through PikAV, and a thioesterase (TE). Two different TEs, TEI and TEII, determine the production of PKM or MTM. Post-production modification occurs after the synthesis of PK backbone. Glycosylation by DesVII and hydroxylation by PikC result various macrolides productions in *S. venezuelae*

of actinorhodin (ACT) and salinomycin productions from *Streptomyces coelicolor* and *Streptomyces albus,* respectively [32], were also suggested by the addition of triclosan, which repressed activities of enoyl-acyl carrier protein reductase of FAS gene. However, this strategy has some drawbacks. First, additions of antibiotics such as triclosan are impractical and unreasonably expensive in real application [19]. In addition, their clearance during purification generates another problem. As a result, controlling FAS gene expression may be a better strategy to reduce FA synthesis. Another general, the so-called "pull" method to redirect acyl-CoA fluxes from FAS into PKS, is to overexpress an entire PKS gene cluster [20], or by manipulations of transcriptional regulators [34]. But, PKS gene clusters are very bulky, which is hard to overexpress.

Enhancements of PK 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 [27, 41]. Glycolysis is a major source of acyl-CoA precursor productions [2, 3, 22]. However, as there are limits to how much antibiotics could be produced from one nutrient source, [12, 24] 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 (Fig. 1a). 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 three direct products of BCDH are further metabolized by acyl-CoA dehydrogenase (ACAD) [25, 31], or are used in synthesis of branched chain fatty acids (BCFA) [35]. It was previously reported that 50% of acetyl-CoA for ACT biosynthesis was supplied from the BCAA degradation pathway [31], and about 52 fold increase in ACT production was observed with BCDH overexpression mutant [10]. 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 [25, 42].

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 [27].

We were interested in how BCAA catabolism would affect pikromycin (PKM) production in *S. venezuelae*, as PKM is biosynthesized using six methylmalonyl-CoAs and one malonyl-CoA [23, 36] by PKS genes (Fig. 1b), and many previous reports on PKM overproduction focused on the manipulations of transcriptional regulators such as *wblA* [37], *afsR*, and *metK* [17] rather than actual metabolic enzymes participating in the biosynthetic or degradation pathways. A simple solution to investigate the effects of BCAA catabolism on the PKM production is to make net fluxes of precursor pools positive by the overexpression of the key enzymes enhancing 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.

Materials and methods

Bacterial strains, plasmids, and culture conditions

All strain information and plasmids used in this study are listed in Table 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.

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. For selection of *S. venezuelae* mutants and conjugates, 8 μg/mL thiostrepton and

Table 1 A list of bacterial strains and plasmids used in this study

Strains or plasmids	Descriptions	References
Streptomyces strains		
Streptomyces venezuelae ATCC15439	Wild type (WT) strain	[39]
$SVEN_{1220KASIII}$	KASIII repression mutant of *Streptomyces venezuelae* ATCC15439	This study
$SVEN_{BCDH}$	*Streptomyces venezuelae* ATCC15439 carrying pIBR25BCDH	This study
$SVEN_{ACAD}$	*Streptomyces venezuelae* ATCC15439 carrying pIBR25ACAD	This study
$SVEN_{MCM}$	*Streptomyces venezuelae* ATCC15439 carrying pIBR25MCM	This study
$SVEN_{BCMC}$	*Streptomyces venezuelae* ATCC15439 carrying pIBR25BM	This study
E. coli strains		
DH5α	A host for gene manipulations	Invitrogen
ET12567	A host for transferring plasmids into *Streptomyces* sp.	ATCC
Plamids		
pIBR25	SCP2* origin, ampicillin and thiostrepton marker	[40]
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	[32]
pIBR25KASIII	pIBR25 containing KASIII in *Pst*I/*Xba*I sites	This study
p1220KASIII	SuperCos-1 containing KASIII with a promoter of SCO1214 and 5′ UTR of SCO2078	This study
pIBR25BCDH	pIBR25 containing BCDH in *Xba*I/*Eco*RI sites	This study
pIBR25ACAD	pIBR25 containing ACAD in *Bam*HI/*Pst*I sites	This study
pIBR25MCM	pIBR25 containing MCM in *Eco*RI/*Hind*III sites	This study
pIBR25BM	pIBR25 containing BCDH in *Xba*I/*Eco*RI sites and MCM 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

50 μg/mL apramycin were used. 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 pellets after pikromycin extractions were dried in an oven at 60 °C for dry cell weight (DCW) measurements.

Escherichia coli DH5α was cultured at 37 °C. *E. coli* ET12567 and *Streptomyces* strains were cultured at 30 °C, 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.

Strain construction

All primers and their sequences are listed in Table S1. 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.

Promoter and 5′ UTR for repression of KASIII were selected from *S. coelicolor*, which was described in the previous study [40]. 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), BCDH (SCO3815-3817), ACAD (SCO2279), and MCM (SCO4869), 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 in pIBR25PikC. ermE*P and *pikC* were PCR amplified together 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 [9].

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.

Pikromycin extraction and quantification

On day 3 of *S. venezuelae* cultures, 40 mL culture broths were harvested and supernatants were collected by centrifugation at 2840×*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 [39]. 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 5 mM ammonium acetate.

Results and discussion

Impact of valine supplementation on pikromycin production

Catabolism of BCAA results in various low molecular weight acyl-CoA (Fig. 1a). Valine can possibly produce three CoA compounds, acetyl-CoA, propionyl-CoA, and methylmalonyl-CoA. Thus, we focused on the 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 tends to decrease cell mass (Fig. 2a). The maximum PKM titer, 22.6 mg/L, was observed at 2 mM valine, which was 1.2 folds more than that without valine supplementation (Fig. 2b), and 1.7 fold increase in production per cell mass (Fig. S1).

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* [38]. In *Streptomyces ambofaciens*, excess valine resulted in ammonium formation and accumulation, reducing cell mass and spiramycin production yields [16]. Reduced cell mass 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 [5, 8, 29]. 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 [29], so that an optimal valine feeding concentration is required. Since 2 mM valine supplementation resulted in some increase in PKM production,

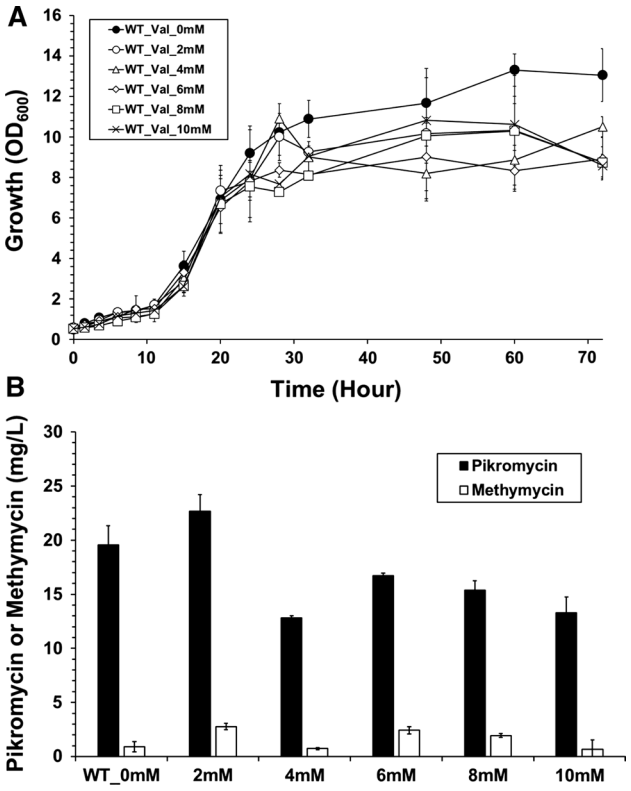


Fig. 2 **a** Growth measured by O.D., and **b** pikromycin and methymycin productions with valine supplementations from 2 to 10 mM to cultures of WT *S. venezuelae* strains

it appeared that there were still rooms to utilize BCAA degradation pathways by modulating metabolic enzymes, and/or regulators to increase PKM production.

BCAA catabolism and pikromycin productions

When BCAAs are catabolized into acyl-CoA compounds via BCDH, they are used for either synthesis of BCFA or PK secondary metabolites. KASIII for BCFA [7] and ACAD for short chain acyl-CoAs [42] 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 [33], reduction of KASIII expression 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 [40].

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. 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 [15]. $P_{SCO1214}R_{SCO2078}$ 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 (Fig. S2a).

The growth of $SVEN_{1220KASIII}$ slightly decreased compared to that of the wild type, measured by cell optical density (OD) (Fig. S2b), which was also confirmed by DCW measurement [i.e., $DCW_{1220FabH} = 6$ g/L, which is about 83% of the control (Fig. S2c)]. In contrast, PKM titer of $SVEN_{1220KASIII}$ decreased more dramatically, by almost a half (Fig. S2d). Previous reports indicated that cell mass at the time of secondary metabolite production is very important, and severe decrease in cell mass may also decrease the production of secondary metabolites [33]. Decrease in PKM production from SVEN1220KASIII can be such case. 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.

To increase metabolic flux towards methylmalonyl-CoA synthesis, we used push and pull strategy by overexpressing BCDH ($SVEN_{BCDH}$) and ACAD ($SVEN_{ACAD}$). DCW among $SVEN_{1220KASIII}$, $SVEN_{BCDH}$, and $SVEN_{ACAD}$ were almost identical (i.e., about 7 g/L DCW) (Fig. 3a). The maximum concentration of PKM was about 25 mg/L, which was 1.3 fold increase compared to the WT (Fig. 3b). Increase in PKM production due to BCDH or ACAD overexpression were almost identical. Overexpression of the two well-known positive regulators *metK* and *afsR* produced about 1.5 and 2 mg/L of PKM, respectively, which were 1.6 fold and 2.6 fold increase of the WT [17]. Deletion of antibiotic biosynthesis down-regulator, *wblA*, increased PKM production from 0.2 to 0.6 mg/L, which was 3.5 fold increase [37]. It would be difficult to compare our data with the previous

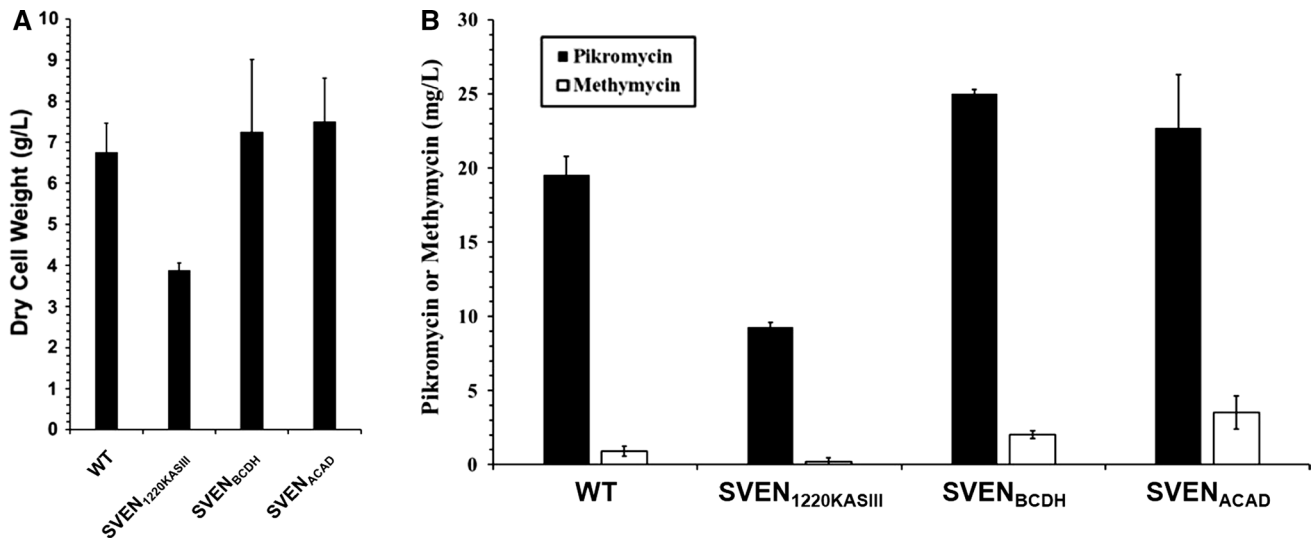


Fig. 3 **a** Dry cell weights of, and **b** Pikromycin and methymycin productions from $SVEN_{1220KASIII}$, $SVEN_{BCDH}$, and $SVEN_{ACAD}$. WT *S. venezuelae* was included as a control

reports due to the differences in culture and production scale. Although our result was a rather small increase in fold changes compared to the previous studies from other groups, such mutations in transcriptional regulators do not generally affect the activity of other metabolic enzymes, so that they can be independently and easily combined with our mutants (data not shown).

One possible explanation of smaller increase could be a low possibility 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 [28, 43]. Therefore, there is a lower probability of methylmalonyl-CoA synthesis for PKM, compared to acetyl-CoA synthesis from BCAA catabolism 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.

Double overexpression mutant of BCDH and MCM

A double overexpression mutant of BCDH and MCM ($SVEN_{BCMC}$) 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. Overexpression of MCM overproduced erythromycin by about 1.4–1.64 folds from *Saccharopolyspora erythraea* [26]. 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 $SVEN_{MCM}$ yielded almost the same DCW, about 7 g/L (Fig. S3a). The cell mass of $SVEN_{MCM}$ 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 $SVEN_{MCM}$, 33.9 mg/L, which was 1.74 fold increase compared to that of the WT.

Surprisingly, PKM titer of $SVEN_{BCMC}$ decreased down to 25.0 mg/L (Fig. S3b), which was almost the same level as that of $SVEN_{BCDH}$. To further examine a reason for such a decrease, we performed more detailed HPLC and LC–MS analysis. Three new compound peaks were detected from chromatograms of HPLC (Fig. 4), and four from TSQ-MS analysis (Fig. 5). The three newly detected compounds from HPLC were collected and further analyzed by MS/MS.

According to MS/MS analysis of the three unidentified compounds from $SVEN_{BCMC}$ culture extracts, the three peaks were 486.45 m/z, 470.40 m/z, and 510.53 m/z, identified as $[novamethymycin + H]^+$ (Fig. S4a), $[neomethymycin + H]^+$ (Fig. S4b), and $[narbomycin + H]^+$ (Fig. S5a), respectively. Novapikromycin was also detected from crude extract of PK antibiotics from $SVEN_{BCMC}$ (Fig. S5b). Novaforms are C12 hydroxylated forms of MTM and PKM. In $SVEN_{BCMC}$, fair amounts of deoxy forms of PK antibiotics were accumulated, which may also explain the decrease in the PKM production. Based on the TSQ-MS data, macrolides were quantified to calculate total production.

Despite the reduction of PKM production in $SVEN_{BCMC}$ compared to that of $SVEN_{MCM}$, total macrolide productions

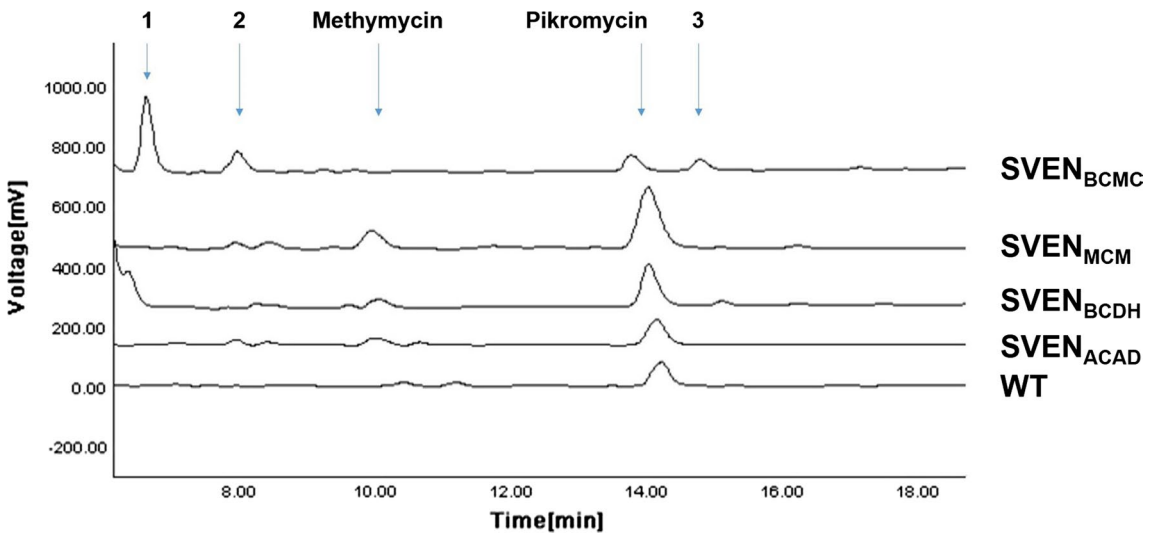


Fig. 4 HPLC chromatograms from supernatant extracts of the WT, $SVEN_{1220KASIII}$, $SVEN_{BCDH}$, and $SVEN_{ACAD}$. Previously unidentified peaks at (1) 6.1 min, (2) 7.4 min, and (3) 14.5 min were observed in the extracts of $SVEN_{BCMC}$ cultures

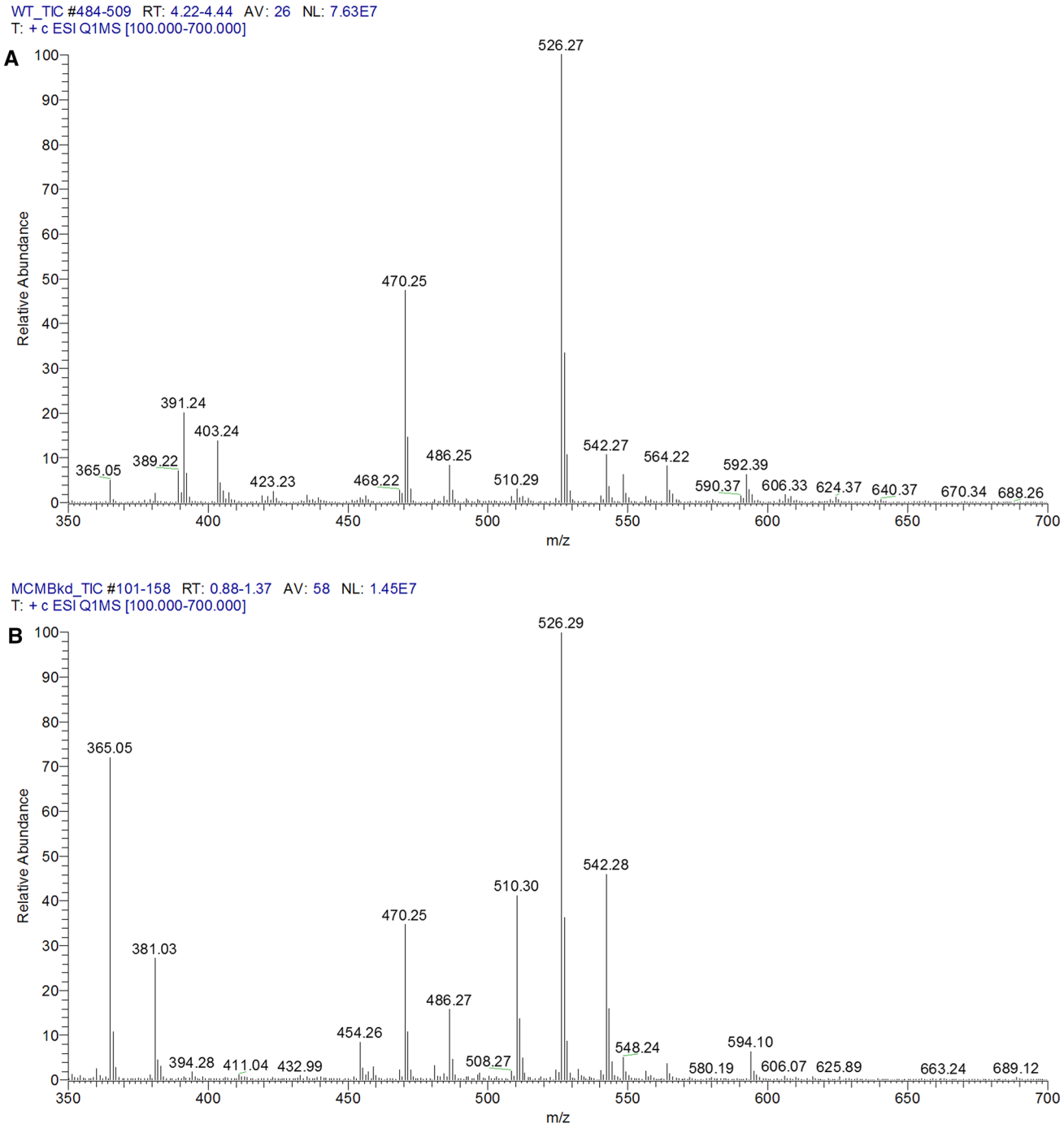


Fig. 5 Total ion chromatogram of TSQ-MS analysis from extracts of **a** WT and **b** $SVEN_{BCMC}$. Total four new metabolite peaks were identified

increased on the other hand (Fig. 6a). Total 43 mg/L of macrolides were produced from $SVEN_{BCMC}$, which was about 2.2 fold increase compared to that of the WT. Considering error ranges, it was about the same as that of $SVEN_{MCM}$. PKM and MTM derivatives, including their deoxy forms, increased dramatically, taking a huge portion in macrolides production (Fig. 6b).

Total macrolides production per biomass of $SVEN_{BCMC}$ was increased by 2.3 fold of the WT, which was about 7.41 mg/L/g_{cell} (Fig. S6a). Interestingly, PKM production per biomass was about the same among the five strains, considering their error ranges (Fig. S6b). Such results indicated that supplementation of acyl-CoA precursors from metabolism was no longer a bottleneck within $SVEN_{BCMC}$.

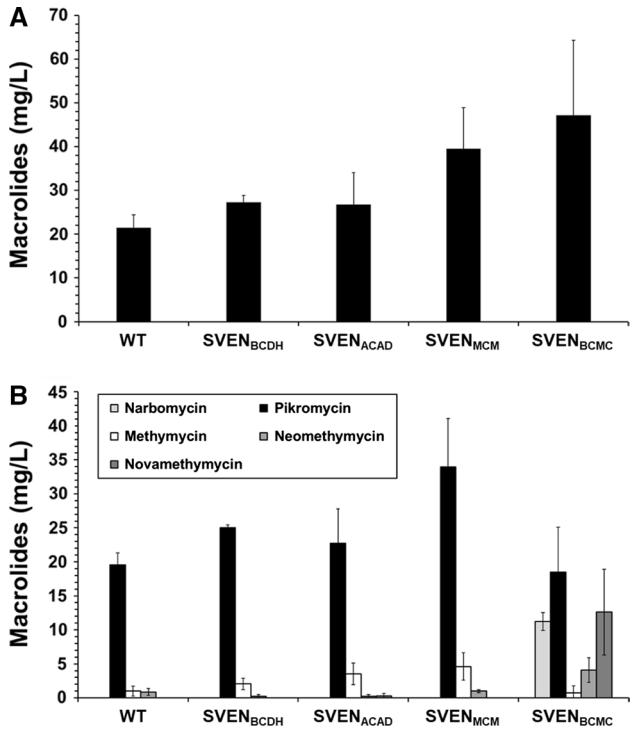


Fig. 6 **a** Total macrolide productions and **b** product profiles from WT, SVEN$_{BCDH}$, SVEN$_{ACAD}$, SVEN$_{MCM}$, and SVEN$_{BCMC}$

But, activities of *pikC*, cytochrome P450 monooxygenase, became a new bottleneck.

pikC is responsible for production of various PKM and MTM derivatives (Fig. S7). For PKM family, *pikC* recognizes desosamine groups, and adds hydroxyl groups at C12 and C14 positions [13, 30]. The enzyme appears to have somewhat broad substrate specificity, and it alone produces neo and nova form derivatives of PKM and MTM. As a result, the increase in PKM production may also mean the increase in its substrate production for *pikC* as well. It applies the same for MTM. But, *pikC* expression throughout the study was not manipulated according to increasing PKM production. In other words, *pikC* expressions in SVEN$_{BCDH}$, SVEN$_{ACAD}$, SVEN$_{MCM}$, and SVEN$_{BCMC}$ were the same. It was reasonable to assume that there were no sufficient *pikC* available for the hydroxylation of PKM and MTM deoxy forms. Such explanation is not enough, and optimizations in the productions of PKM and MTM would require further detailed studies, but to convert the accumulated deoxy form antibiotics into PKM and MTM, overexpression of *pikC* in SVEN$_{BCMC}$ was attempted.

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 [6], 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 to be 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 PK compounds, *pikC* was shown to hydroxylate many of them in the previous reports, resulting in numerous hydroxylated aminoglycoside products [14]. Taking advantages of its large substrate specificity, *pikC* was mutated to accept various substrates with different amino sugars or types of aglycones [21]. If *pikC* could be mutated to accept narbomycin and nothing else, it would be possible to further maximize the PKM production.

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. [23]. 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 PK antibiotics was emphasized [10, 31]. 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 increase, 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, which were about 2.2 fold increase compared to that of the WT. 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 manipulate 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.

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