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# Improvement of FK506 Production in the High-Yielding Strain *Streptomyces* sp. RM7011 by Engineering the Supply of Allylmalonyl-CoA Through a Combination of Genetic and Chemical Approach S

**SangJoon Mo[1†], Sung-Kwon Lee[2†], Ying-Yu Jin[2], and Joo-Won Suh[2*]**

[1]*Clinical Trial Institute, Biosafety & Validation Center, Dankook University, Choenan 31116, Republic of Korea*
[2]*Division of Bioscience and Bioinformatics, Myongji University, Youngin 17058, Republic of Korea*

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*Corresponding author
Phone: +82-31-330-6190;
Fax: +82-31-336-0870;
E-mail: jwsuh@mju.ac.kr

†These authors contributed equally to this work.

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FK506, a widely used immunosuppressant, is a 23-membered polyketide macrolide that is produced by several *Streptomyces* species. FK506 high-yielding strain *Streptomyces* sp. RM7011 was developed from the discovered *Streptomyces* sp. KCCM 11116P by random mutagenesis in our previous study. The results of transcript expression analysis showed that the transcription levels of *tcsA*, *B*, *C*, and *D* were increased in *Streptomyces* sp. RM7011 by 2.1-, 3.1-, 3.3-, and 4.1-fold, respectively, compared with *Streptomyces* sp. KCCM 11116P. The overexpression of *tcsABCD* genes in *Streptomyces* sp. RM7011 gave rise to approximately 2.5-fold (238.1 μg/ml) increase in the level of FK506 production compared with that of *Streptomyces* sp. RM7011. When vinyl pentanoate was added into the culture broth of *Streptomyces* sp. RM7011, the level of FK506 production was approximately 2.2-fold (207.7 μg/ml) higher than that of the unsupplemented fermentation. Furthermore, supplementing the culture broth of *Streptomyces* sp. RM7011 expressing *tcsABCD* genes with vinyl pentanoate resulted in an additional 1.7-fold improvement in the FK506 titer (498.1 μg/ml) compared with that observed under non-supplemented condition. Overall, the level of FK506 production was increased approximately 5.2-fold by engineering the supply of allylmalonyl-CoA in the high-yielding strain *Streptomyces* sp. RM7011, using a combination of overexpressing *tcsABCD* genes and adding vinyl pentanoate, as compared with *Streptomyces* sp. RM7011 (95.3 μg/ml). Moreover, among the three precursors analyzed, pentanoate was the most effective precursor, supporting the highest titer of FK506 in the FK506 high-yielding strain *Streptomyces* sp. RM7011.

**Keywords:** Allylmalonyl-CoA, FK506, FK506 high-yielding strain *Streptomyces* sp. RM7011, gene overexpression, precursor supplementation

## Introduction

Members of the genus *Streptomyces* are well known as producers of pharmaceutically relevant natural products with wide-ranging biological effects that include antimicrobial, anticancer, and immunosuppressant activities. FK506, also known as tacrolimus, is a 23-membered polyketide macrolide with potent immunosuppressive activity. The immunosuppressive action of FK506 reflects the selective blocking of a subset of $Ca^{2+}$-dependent signaling events that regulate the expression of early activation genes in T cells, including those of the lymphocyte growth-promoting lymphokines interleukin-2 and interleukin-4. Since the first isolation of FK506 from the soil bacterium *Streptomyces tsukubaensis* in 1987 [10, 11], it has been found to be produced by a variety of *Streptomyces* species. FK506 is widely used to prevent graft rejection after organ transplantations [5], and it is also used in a topical preparation for the treatment of atopic dermatitis [6] and is being investigated in the treatment of autoimmune diseases such as rheumatoid arthritis, ulcerative colitis, and Crohn's disease [1, 3]. Owing to the pharmaceutical potential and broad clinical

applications described above, considerable time and effort have been expended to enhance the yield of FK506, and to develop more efficient and economical processes for its industrial production [9, 15]. Recently, engineering the precursor supply for FK506 product has been used to increase the productivity of FK506. In addition, various approaches have been used to increase the productivity of FK506 by using information on the FK506 biosynthetic

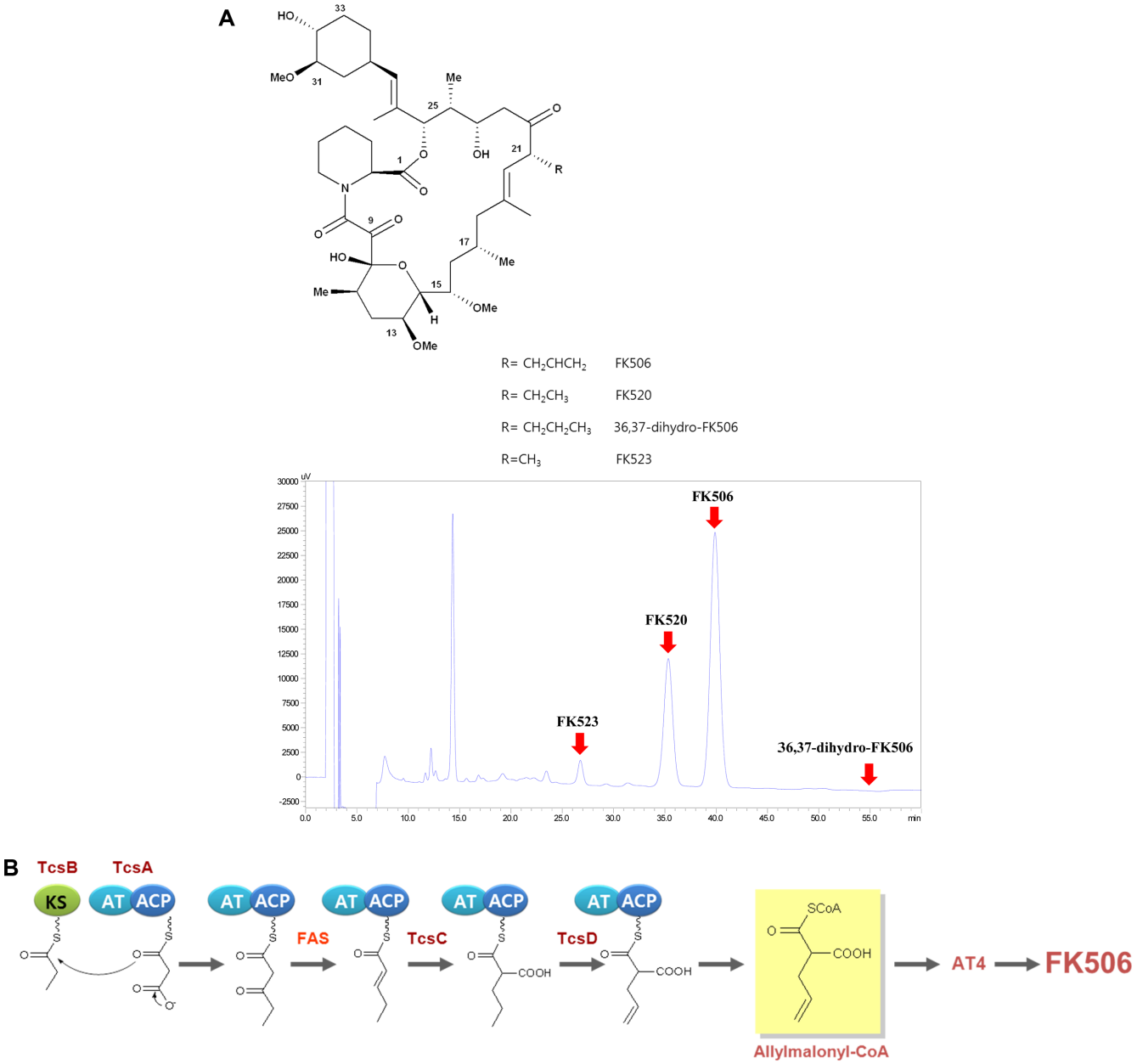


**Fig. 1.** Structures and HPLC chromatogram of FK506, FK520, 36,37-dihydro-FK506, and FK523, and the biosynthetic pathway of AM-CoA in *Streptomyces* sp. KCCM 11116P.

(**A**) Chemical structure and HPLC trace of FK506 and its congeners. The allyl moiety at C21 of FK506 is replaced by an ethyl, propyl, and methyl moiety in FK520, 36,37-dihydro-FK506, and FK523, respectively. The chromatogram from HPLC analyses monitoring absorbance at 205 nm of crude extracts of KCCM 11116P grown in R2YE liquid medium shows that the FK523 (retention time: 27 min), FK520 (35 min), and FK506 (40 min) peaks are inclusive to KCCM 11116P, and 36,37-dihydro-FK506 (55 min) is only in a trace amount when KCCM 11116P is grown in R2YE medium. (**B**) Biosynthetic pathway of AM-ACP catalyzed by TcsA, B, C, and D. The AM-ACP is presumed to be converted to AM-CoA by an unidentified ACP:CoA transacylase-like enzyme. AM-CoA is subsequently loaded onto module 4 of FK506 PKS.

pathway and full genome sequence. These results can be the model to improve the secondary metabolite production, combining the previously described precursor feed-up and the biosynthetic genes amplification. This strategy fits quite well with the new trends in industrial secondary metabolites production, where synthetic biology, random mutagenesis, and rational engineering are combined successfully [2].

FK506 has a structurally distinguished, unique allyl moiety at carbon 21, which is derived from allylmalonyl (AM)-CoA, as compared with other congeners (FK520 from ethylmalonyl-CoA, FK523 from methylmalonyl-CoA, and 36,37-dihydro-FK506 from propylmalonyl-CoA) (Fig. 1A). Besides identifying the four *tcs* genes (*tcsA*, *tcsB*, *tcsC*, and *tcsD*) supporting AM-CoA biosynthesis encoded in the *tcs* subcluster, the enzymatic functions were also established through the in vivo/in vitro reconstruction of the biosynthetic pathway [14]. TcsA/B, encoding small diketide synthase, putatively gives rise to the β-ketopentyl-acyl carrier protein (ACP) product, which cooperates with the endogenous fatty acid synthase system to convert *trans*-2-pentenyl-ACP. Consecutive modifications of the ACP-bound pentenyl group, including reductive carboxylation by TcsC, as well as dehydrogenation by TcsD, form ACP-tethered allylmalonate, which is thioester migrated to AM-CoA before acyltransfer by the AM-specific acyltransferase domain in module 4 of the FK506 polyketide synthase (Fig. 1B) [14]. Recently, Chen *et al*. [4] reported the enhancement of FK506 titer in an FK506-producing strain by overexpression of a pathway-specific gene encoding the biosynthesis of methoxymalonyl (MOM)-ACP and AM-CoA extender unit. They suggested that FK506 production can be further improved by coenhancing the biosyntheses of MOM-ACP and AM-CoA without the positional effect, by using the two integrase-based recombination systems in *S. tsukubaensis* [4].

In a previous study, we developed an FK506 high-yielding strain, *Streptomyces* sp. RM7011 (RM7011), from the wild-type *Streptomyces* sp. KCCM 11116P (KCCM 11116P) by random mutagenesis. Strain RM7011 showed an 11.6-fold (94.2 μg/ml) increase of FK506 production compared with KCCM 11116P [15]. Recent progress from understanding the overexpression of AM-CoA biosynthetic genes shows huge potential to improve FK506 production in FK506 high-yielding strains by increasing the supply of precursors. To achieve this, transcript expression analysis, genetic manipulation, and chemical complementation have been used, including semi-quantitative reverse transcription (RT)-PCR, gene overexpression, and precursor feeding.

In the present study, in order to elucidate the cellular mechanisms behind the FK506 yield improvement in our FK506 high-yielding strain, transcriptional analysis of RM7011 and its parental strain, KCCM 11116P, was performed during growth of the cells, using semi-quantitative RT-PCR. Based on this, we show the importance of the expression of *tcsA*, *B*, *C*, and *D* in the production of FK506 in RM7011. In particular, precursor supplementation of the AM-CoA biosynthetic pathway has caused a remarkable effect on enhancing the FK506 titer in the *tcsABCD*-overexpressing *Streptomyces* sp. RM7011 (RM7011/pSKO).

## Materials and Methods

### Bacterial Strains, Plasmids, Primers, and Culture Conditions

All bacterial strains and plasmids used in this study are listed in Table S1. Primers are listed in Table S2. Strain KCCM 11116P was isolated and developed at our laboratory in previous study [15]. The FK506 high-yielding strain, RM7011, was derived by random mutagenesis from strain KCCM 11116P in a previous study [15]. Luria-Bertani (LB) agar and liquid media were used to grow *Escherichia coli* [19]. Spore generation and seed culture of strains for FK506 production were done as described previously [15]. For measurement of the FK506 production of KCCM 11116P and RM7011, 50 mg of mycelium from the seed culture was inoculated into a baffled 250 ml flask containing 50 ml of R2YE medium with or without thiostrepton at pH 7.2, and cultivated for 6 days at 28°C and 180 rpm. To isolate the total RNA, spore solutions of KCCM 11116P and RM7011 were inoculated into 50 ml of R2YE medium and cultivated at 28°C and 180 rpm. Thirty-six hours after inoculation, 50 mg of mycelium was inoculated into 50 ml of R2YE and cultivated at 28°C and 180 rpm for 3 days, and the mycelium was collected at selected time intervals. Ampicillin (50 μg/ml) and thiostrepton (25 μg/ml), all obtained from Sigma-Aldrich (St. Louis, MO, USA), were added to the agar media as required. For analysis of FK506 in *tcsABCD*-overexpressed KCCM 11116P and RM7011, all strains (KCCM 11116P, KCCM 11116P/

**Table 1.** Effect of *tcsABCD* gene overexpression on FK506 production in strains KCCM 11116P and RM7011.

| Characterization | KCCM 11116P | KCCM 11116P/pSE34 | KCCM 11116P/pSKO | RM7011 | RM7011/pSE34 | RM7011/pSKO |
|---|---|---|---|---|---|---|
| FK506 production (μg/ml)[a] | 8.7 ± 0.2 | 8.3 ± 0.4 | 21.2 ± 0.3 | 95.3 ± 0.2 | 92.8 ± 0.7 | 238.1 ± 0.7 |

[a]Cultures were grown for 6 days in R2YE medium. The cell-free broths were collected in a flask by vacuum filtration and then extracted with ethyl acetate for HPLC analysis. An authentic FK506 standard was used to construct a calibration curve of FK506 by HPLC analysis. Data are presented as the mean value ± standard deviation ($n$ = 3).

pSE34, KCCM 11116P/pSKO, RM7011, RM7011/pSE34, and RM7011/pSKO) were inoculated into 20 ml of R2YE medium and cultivated for 48 h at 28°C and 180 rpm, and then 500 µl of each seed culture broth was inoculated into 50 ml of R2YE medium with or without thiostrepton and cultivated for 6 days at 28°C and 180 rpm.

**Isolation of mRNA and Gene Expression Analysis by RT-PCR**

For RNA isolation, KCCM 11116P and RM7011 were grown on R2YE medium for 72 h. Mycelia were harvested and suspended in RNAprotect Bacteria Reagent (Qiagen, Valencia, CA, USA), and suspensions were kept at −80°C. RNA was isolated with an RNeasy mini kit (Qiagen), according to the manufacturer's instructions. RNase-free DNase (Qiagen) was used to eliminate any contaminant DNA from the RNA sample. The quantity and integrity of the RNA preparations were determined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis. RT-PCR was carried out with the Qiagen OneStep RT-PCR kit, using 1 µg of total RNA as a template. The conditions were as follows: for cDNA synthesis, 50°C for 30 min, followed by 95°C for 15 min; amplification, 35 or 38 cycles of 97°C for 1 min, 60–70°C (depending on the set of primers used) for 1 min, 74°C for 1 min, and 75°C for 5 min. The cycle number for each gene was optimized in order to obtain enough visibility of the RT-PCR band, to ensure that amplification was in the linear range, and to ensure the results were semi-quantitative [7, 16]. The primers were designed to generate PCR products of approximately 500–660 bp, and each amplified product was verified by DNA sequencing. Negative control experiments were carried out in the absence of reverse transcriptase to check for DNA contamination in the RNA preparations. Primers targeting 16S rRNA were used as positive controls for RNA quality [17].

**Quantification of RT-PCR Products by Image Analysis**

After RT-PCR amplification was complete, 20 µl aliquots of the reaction products (in a total volume of 50 µl) were electrophoresed on a 1.5% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml), and images of the samples on the electrophoresed gels were captured by using the Bio-Rad Gel Doc XR$^+$ system with exposure time of 2 sec. Once the images had been acquired, optimization to reduce noise or background was performed. Image analysis of the one-dimensional agarose gels was carried out after electrophoresis to achieve relative quantification of the RT-PCR products, using Image Lab software ver. 5 (Bio-Rad, Hercules, CA, USA). Relative amounts of amplified products were the comparison of the experimental data with a standard calibration curve ($y = 1.0638x + 0.0201$, $r^2 = 0.996$) obtained using purified DNA at varying concentrations [7, 17, 18]. All data were calculated as the average from at least three replicates.

**Analysis of FK506 Production**

The level of FK506 production was determined by high performance liquid chromatography (HPLC). Fifty-milliliter samples of culture broth were extracted twice with an equal volume of ethyl acetate. The organic extract was dried using a rotary evaporator under reduced pressure, and then dissolved in 0.2 ml of methanol for use in HPLC analysis. Samples were loaded onto a Supelco Discovery HS C18 column (Supelco Analytical, Bellefonte, PA, USA), which was maintained at 50°C. The mobile phase used for isocratic elution was composed of water, acetonitrile, methyl-*tert*-butyl ether, and phosphoric acid (58.29:34.4:7.29:0.02 (v/v/v/v)). The flow rate was 1 ml/min, and detection was carried out with a UV detector at 205 nm. The HPLC analysis method used for the determination of FK506 production was modified from previously reported methods [12, 13]. The HPLC chromatogram of FK523, FK520, FK506, and 36,37-dihydro-FK506, which eluted with a retention time of approximately 27, 35, 40, and 55 min, respectively, is shown in Fig. 1A. The elution time was compared with the standard and the compound was determined. Authentic FK506 and FK520 (Sigma-Aldrich) were used to create a calibration curve.

**Construction of Plasmids for Overexpression of AM-CoA Biosynthetic Genes**

For overexpression of the *tcsABCD* genes in KCCM 11116P and RM7011, the high-copy number *E. coli-Streptomyces* shuttle vector pSE34 containing the strong *ermE** promoter plus a thiostrepton resistance marker was used [20]. The overexpression plasmids were constructed by PCR amplification of the fragments of the *tcsABCD* genes from the genomic DNA derived from KCCM 11116P (GenBank Accession No. HQ696504.1). The primer pair EXtcsABCDL/EXtcsABCDR was designed to PCR amplify the DNA fragments containing the *tcsABCD* genes (see Table S2 in the Supplementary materials), and the primer pair for the PCR amplification of the *tcsABCD* genes was designed to contain the natural ribosomal binding sites of the *tcsABCD* genes. PCR fragments (6.2 kb for *tcsABCD* genes) were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA) and then sequenced. After digestion with the FbaI and XbaI restriction enzymes, the fragments were cloned into the BamHI and XbaI sites of pSE34, yielding pSKO. pSKO was then introduced into KCCM 11116P and RM7011 by protoplast transformation, respectively, following an established protocol [8], and thiostrepton-resistant transformants were selected as the resulting mutants, KCCM 11116P/pSKO and RM7011/pSKO.

**Effects of AM-CoA Precursors on FK506 Production in RM7011**

Seed cultures of RM7011 were grown in R2YE medium for 48 h at 28°C and then a 1% (v/v) inoculum was used for 50 ml liquid culture of the same medium. Vinyl acetate, vinyl propionate, and vinyl pentanoate (Sigma-Aldrich) were added to a final concentration of 5 mM in R2YE medium supplemented with 5 mM Tween 80. The production cultures were grown for 6 days at 28°C as described previously [13, 15]. For analysis of time course for FK506 production by both the expression of *tcsABCD* genes and the

addition of exogenous 5 mM vinyl pentanoate, RM7011/pSKO was fed with 5 mM vinyl pentanoate in R2YE medium supplemented with 5 mM Tween 80, and then duplicated samples (50 ml) of fermentation broth were collected to measure FK506 production every 24 h for 6 days after the main culture inoculation and extracted as described previously [13, 15].

## Results and Discussion

### Transcription Level Analysis of *tcsA*, *tcsB*, *tcsC*, and *tcsD* Genes in KCCM 11116P and RM7011

In order to study the difference in expression profiles of *tcsABCD* transcripts between KCCM 11116P and RM7011, semi-quantitative RT-PCR was used to quantify the amount of transcripts of these genes. The total RNA was isolated from KCCM 11116P and RM7011 after 16, 20, 24, 48, and 72 h of growth, to compare the expression levels of the four AM-CoA biosynthetic genes. The level of the unrelated, constitutively expressed 16S rRNA gene transcript, assayed as a control for RNA quality, integrity, and loading levels, did not vary between culture times. After electrophoresis, the band intensities of the PCR products of *tcsABCD* genes in KCCM 11116P were

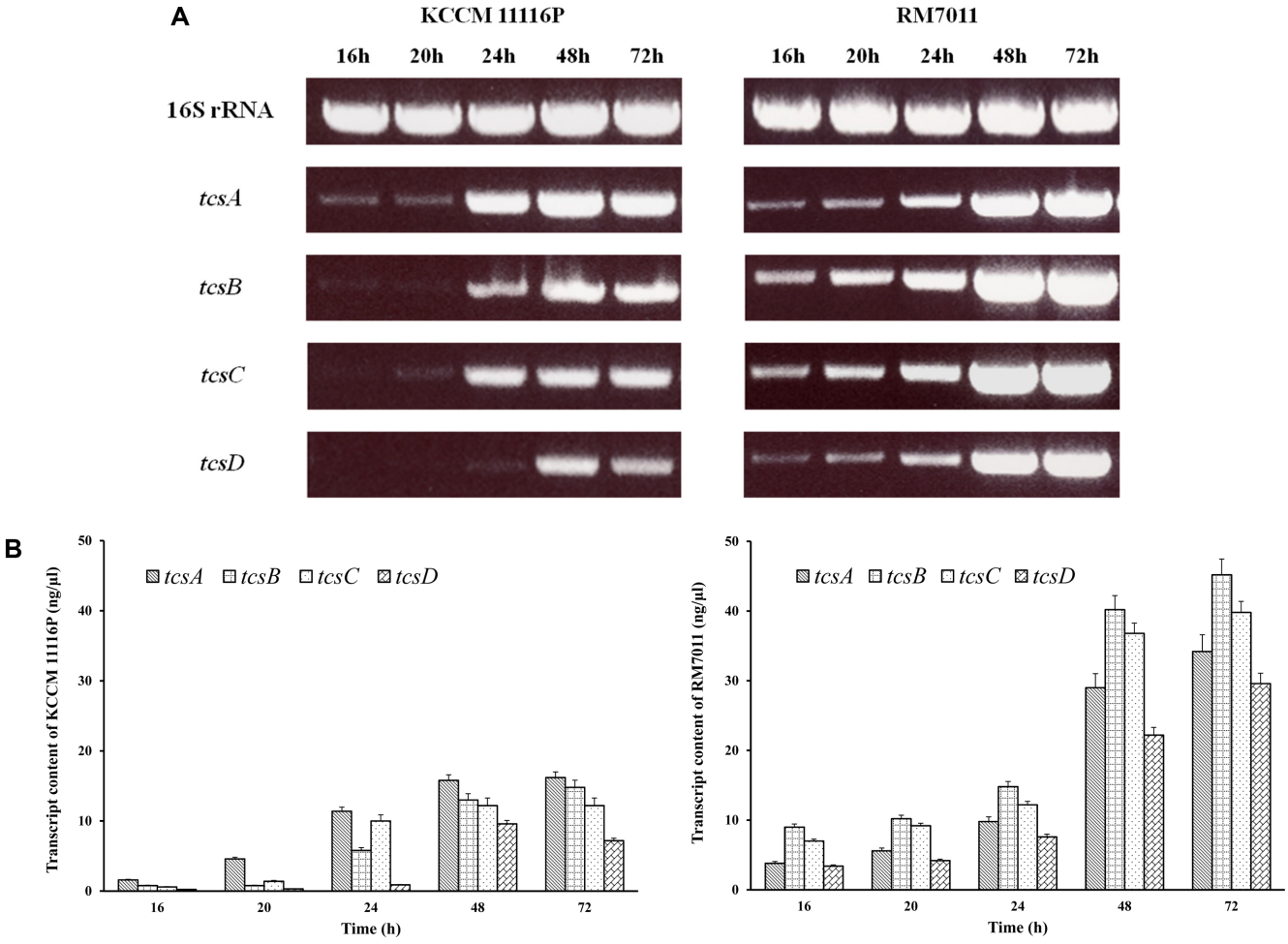


**Fig. 2.** Transcriptional analysis of the AM-CoA biosynthetic genes in KCCM 11116P and RM7011.
(**A**) Semi-quantitative RT-PCR analysis of the transcription level of AM-CoA biosynthetic genes in KCCM 11116P and RM7011 (after being fermented for 16, 20, 24, 48, and 72 h). Reverse transcribed 16S rRNA was amplified as a control for RNA integrity and loading levels. (**B**) The transcription expression levels of *tcsA*, *tcsB*, *tcsC*, and *tcsD* were analyzed, with the intensity of each PCR band being measured by the calibration curve method. Relative amounts of transcript levels of *tcsABCD* were determined by calculation of the average signal intensity of each PCR band in relation to that of a known quantity standard (NoLimits 500 bp DNA fragment). In the calibration curve method, a series of external DNA standard solutions (ranging 0–0.5 ng/μl) were prepared and measured. The measured signal intensity of each together with the equation for the line generated in the DNA standard curve should allow determination of the relative PCR band concentrations in each sample. Downward diagonal, *tcsA*; Dotted grid, *tcsB*; Spot, *tcsC*; Brick hatched pattern, *tcsD*. All error bars represent the standard deviation ($n$ = 3).

compared with those of RM7011 (Fig. 2A). Relative quantification yields the transcription levels of target genes by comparison with DNA standards (NoLimits 500 bp DNA fragment; Thermo Fisher Scientific, Waltham, MA, USA), using a calibration curve. The curve is generated by using serially diluted standards of know concentrations and produces a linear relationship between signal intensity and amount of DNA standards. The results are shown in Fig. 2B. The transcription levels of *tcsA*, *tcsB*, *tcsC*, and *tcsD* at 72 h in RM7011 was an increase of approximately 2.1- ($P < 0.07$), 3.1- ($P < 0.05$), 3.3- ($P < 0.04$), and 4.1- ($P < 0.05$) fold compared with the KCCM 11116P, respectively (Fig. 2B). On the other hand, KCCM 11116P showed transcription levels of 52.6%, 67.3%, 69.3%, and 75.7% lower than those of the RM7011 for the four genes, respectively. The transcription levels of three other FK506 biosynthesis genes, *fkbM*, *fkbB*, and *fkbN*, were also analyzed by RT-PCR in KCCM 11116P and RM7011. These three genes were expressed throughout growth and tended to increase in expression level, but no remarkable increase was observed by the FK506 high-yielding strain (data not shown). These results suggested that the mechanism of high production of FK506 in high-yielding strain RM7011 was closely related to the increase in intracellular level of AM-CoA, probably due to the high-level expression of the AM-CoA biosynthetic genes.

### Effects of *tcsABCD* Genes Overexpression on FK506 Production

The above-mentioned transcriptional analysis showed an inseparable relationship between FK506 production and the transcriptional levels of the AM-CoA biosynthetic genes. To confirm this result, we investigated the effects of the above-mentioned AM-CoA biosynthetic genes on the production of FK506 by overexpression of the *tcsABCD* genes in KCCM 11116P and RM7011. The results are given in Table 1. KCCM 11116P/pSKO showed an approximately 2.4-fold increase in the yield of FK506 compared with that of KCCM 11116P (8.7 μg/ml). In particular, RM7011/pSKO showed an approximately 2.5-fold increase in yield of FK506 compared with that of the RM7011 (95.3 μg/ml) (Table 1). These results also support the hypothesis that AM-CoA is among the major rate-limiting factors for high production of FK506 in KCCM 11116P and RM7011.

### Enhanced FK506 Production by Vinyl Pentanoate Supplementation in RM7011 and RM7011/pSKO

In order to increase the intracellular level of AM-CoA and improve FK506 production, three precursors of AM-CoA biosynthetic pathway were introduced into RM7011 and RM7011/pSKO: vinyl acetate, vinyl propionate, and vinyl pentanoate. Interestingly, of the three precursors examined, feeding with vinyl pentanoate to the RM7011 gave rise to approximately 2.2-fold increase in the levels of FK506 production compared with that of the non-feeding condition (94.4 μg/ml), whereas vinyl acetate and vinyl propionate led to approximately 1.2- and 1.6-fold increase in the levels of FK506 production per milliliter, respectively,

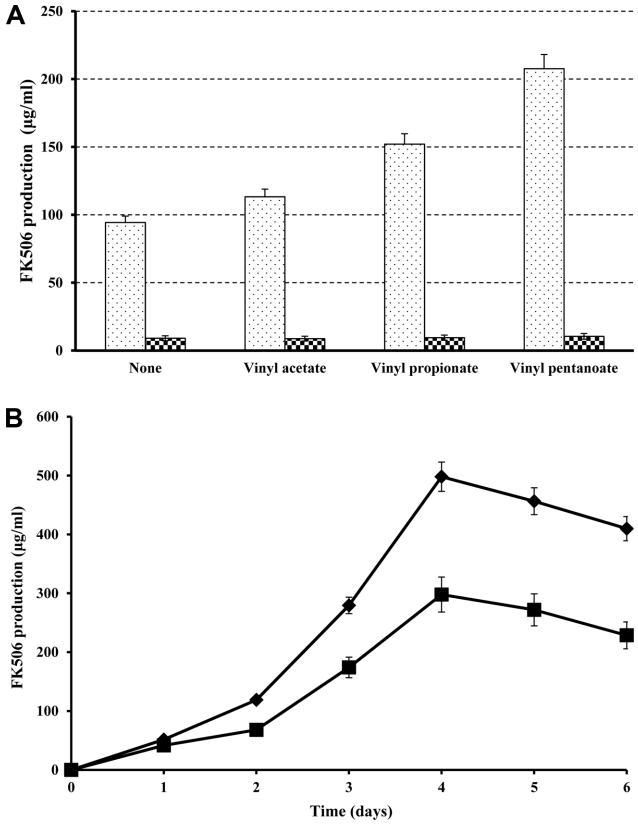


**Fig. 3.** Effect of precursor supply on the production of FK506 by KCCM 11116P, RM7011, and RM7011/pSKO in R2YE medium supplemented with 5 mM Tween 80.

(**A**) Effects of three precursors on FK506 titer in KCCM 11116P and RM7011 fermentation supplemented with 5 mM Tween 80. Spot pattern, the FK506 production in RM7011 by supplementation of vinyl acetate, vinyl propionate, and vinyl pentanoate; Checkerboard pattern, the FK506 production in KCCM 11116P by supplementation of vinyl acetate, vinyl propionate, and vinyl pentanoate. (**B**) Time course for FK506 production by both the overexpression of *tcsABCD* genes and the addition of exogenous 5 mM vinyl pentanoate in R2YE medium supplemented with 5 mM Tween 80. Solid square, RM7011/pSKO without vinyl pentanoate; solid diamond, RM7011/pSKO with vinyl pentanoate. All error bars represent the standard deviation ($n = 3$).

compared with that of the non-feeding condition (Fig. 3A). However, the addition of the three precursors to KCCM 11116P did not affect the FK506 titers (Fig. 3A), suggesting that the exogenously added precursors were not converted into AM-CoA under low-level transcription of *tcsABCD* genes in KCCM 11116P. To further improve the production of FK506 by the combination approach of *tcsABCD* genes overexpression and vinyl pentanoate supplementation, RM7011/pSKO was monitored throughout a 6-day incubation period with 5 mM vinyl pentanoate supplementation. The addition of vinyl pentanoate in the culture of RM7011/pSKO resulted in an additional 1.7-fold improvement in the FK506 titer as compared with RM7011/pSKO at 4 days (Fig. 3B). This result supports the hypothesis that exogenous vinyl pentanoate is catabolized to pentanoate by lipase, and that pentanoate generates pentanoyl-CoA and converts it into AM-CoA. Consequently, these results clearly show that the AM-CoA supply is essential and limiting for FK506 biosynthesis in RM7011, and that of the three different precursors analyzed, the C5 substrate pentanoate is the dominant precursor supporting the highest titer of FK506 in RM7011, even though more detailed mechanisms need to be examined through additional experiments, such as an incorporation analysis using a radioisotope substrate.

In conclusion, the semi-quantitative RT-PCR results in KCCM 11116P and RM7011 showed that the increased transcription levels of AM-CoA biosynthetic genes led to an improvement in the FK506 yields of the FK506 high-yielding strain RM7011. It showed that the level of FK506 production could be easily increased by engineering the level of AM-CoA in RM7011 through a combination of chemical supplementation and genetic approaches. Although AM-CoA has been suggested to be a limiting factor in production of FK506, this study on vinyl pentanoate-supplemented RM7011/pSKO suggested that pentanoate is a key precursor of AM-CoA in increasing the FK506 production. These results show the highest production enhancement over the high-yielding strain, demonstrating that our combined approach could be successfully applied for the rapid development of a high-yielding strain of other target polyketides.

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