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ORIGINAL PAPER

Enhanced FK506 production in *Streptomyces clavuligerus* CKD1119 by engineering the supply of methylmalonyl-CoA precursor

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Abstract FK506 is a 23-membered polyketide macrolide with immunosuppressant activity produced by *Streptomyces* species. The production of FK506 in *S. clavuligerus* CKD1119 (KCTC 10561BP) was improved by enhancing the supply of biosynthetic precursors. This improvement was approximately 2.5-fold (15 mg/l) with the supplementation of 10 m*M* methyl oleate, which is the probable source of acyl-CoAs, to R2YE medium. When the level of FK506 production reached its maximum, the intracellular concentration of methylmalonyl-CoA in *S. clavuligerus* CKD1119 supplemented with methyl oleate was 12.5-fold higher than that of the unsupplemented strain, suggesting that an increased methylmalonyl-CoA level caused the high-level production of FK506. The following three pathways for the production of (2*S*)-methylmalonyl-CoA were evaluated to identify the effective precursor supply pathway that can support the high production of FK506 in *S. clavuligerus* CKD1119: propionyl-CoA carboxylase, methylmalonyl-CoA mutase (MCM), and malonyl/methylmalonyl-CoA ligase. Of the three pathways examined, the MCM pathway supported the highest levels of FK506 production. The expression of MCM in *S. clavuligerus* CKD1119 led to a threefold and 1.5-fold increase in the methylmalonyl-CoA pool and FK506 production, respectively. Supplementing the culture broth of *S. clavuligerus* CKD1119 expressing MCM with methyl oleate resulted in an additional twofold increase in the FK506 titer (17.8 mg/l). Overall, these results show that the methylmalonyl-CoA supply is a limiting factor for FK506 biosynthesis and that among the three pathways analyzed, the MCM pathway is the most effective precursor supply pathway supporting the highest titer of FK506 in *S. clavuligerus* CKD1119.

Keywords FK506 · Macrolide polyketide · Methylmalonyl-CoA · Methyl oleate · Precursor engineering

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Introduction

FK506 (tacrolimus) is a 23-membered polyketide macrolide with immunosuppressant activity that is produced by a variety of *Streptomyces* species [14, 15, 18, 20]. Since the Food and Drug Administration (FDA) approved the use of FK506 for liver transplantation in 1994, FK506 has been licensed as an immunosuppressive drug for use after solid organ transplantation, and it has also shown several other promising therapeutic applications [34]. Due to its pharmacological importance and broad applicability, considerable effort has been expended over the past few decades to elucidate its biosynthetic gene cluster [20, 21] and enhance the yield of FK506. Medium optimization [40] and classical strain improvement methods [13] have been used to increase the FK506 titer.

Polyketides are a large and structurally diverse group of secondary metabolites possessing a wealth of pharmacologically important activity [25]. Polyketide biosynthesis is catalyzed by polyketide synthases (PKSs) in microorganisms and plants using a mechanism similar to that utilized in fatty acid synthesis [8]. Regardless of their structural diversity, polyketides are assembled using several common biosynthetic precursors, which include malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA [3, 8, 11]. In streptomycetes, malonyl-CoA and ethylmalonyl-CoA

are likely to be generated by the carboxylation of acetyl-CoA and butyryl-CoA/crotonyl-CoA, respectively, while numerous pathways give rise to methylmalonyl-CoA [7, 10, 17, 29, 30, 41, 42]. There has been considerable interest in enhancing the yield of polyketides by increasing the supply of precursors [16, 23, 27, 28]. Furthermore, in classically improved industrial polyketide-producing actinomycete strains, the overproduction mechanisms have been found to involve the lengthened expression of biosynthetic genes or a change in precursor metabolism to increase the supply of precursor(s) for biosynthesis [19]. FK506 biosynthesis begins with dihydroxycyclohexane carbonyl-CoA as the starter unit, which is followed by ten successive extension steps for the synthesis of the polyketide chain using four malonyl-CoAs [or two malonyl-CoAs and two methoxymalonyl-ACPs (acyl carrier protein)], five methylmalonyl-CoAs, and possibly one propionylmalonyl-CoA. The linear polyketide chain synthesized by PKS is condensed with pipecolate, followed by cyclization to form the macrolide ring (Fig. 1) [3, 20, 21, 39].

In the study reported here, the level of FK506 production in *S. clavuligerus* CKD1119 was enhanced by promoting the intracellular pool of methylmalonyl-CoA. The methylmalonyl-CoA mutase (MCM) pathway participating in the biosynthesis of methylmalonyl-CoA from succinyl-CoA was introduced to the wild-type strain of *S. clavuligerus* CKD1119 along with the supplementation of methyl oleate to the medium, resulting in an approximately threefold improvement in the FK506 titer. An analysis of the intracellular acyl-CoAs suggested that the increased acetyl-CoA pool by methyl oleate supplementation can be converted efficiently, possibly by succinyl-CoA, to methylmalonyl-CoA via the MCM pathway, which can increase the level of FK506 production. These results demonstrate that engineering the precursor supply and a detailed understanding of the limiting precursor for the target product can assist in the development of a high-producing strain of the target polyketide.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The FK506-producing *S. clavuligerus* CKD1119 (KCTC 10561BP) strain used in this study was obtained from the Korean Collection for Type Cultures (Daejeon, Korea). The *Escherichia coli* strain DH10B used as a host for the cloning experiments [31]. *Escherichia coli* ET12567/pUZ8002 was used as the nonmethylating plasmid donor strain [12] for intergeneric conjugation with *S. clavuligerus* CKD1119. Litmus28 (New England Biolabs, Beverly, MA) was used for subcloning, and integrative *E. coli–Streptomyces* vector pSET152 [1] derivative containing the *ermE** promoter (P_{ermE*}) [32] was used for gene expression in *S. clavuligerus* CKD1119. Spores of *S. clavuligerus* CKD1119 were produced on ISP4 agar plates [33], and a seed culture was prepared in R2YE broth [12]. The production of FK506 was carried out by inoculating a baffled 250-ml flask containing 50 ml of R2YE medium at pH 7.2 with 500 µl of a seed culture suspension and then incubating the suspension on an orbital shaker (180 rpm) for 6 days at 28°C. *Saccharopolyspora erythraea*, *Streptomyces coelicolor* A3(2), and *Salmonella typhimurium* LT2 ATCC 29631 were cultivated in SCM, YEME, and nutrient broth, respectively, for genomic DNA isolation [9, 12, 28].

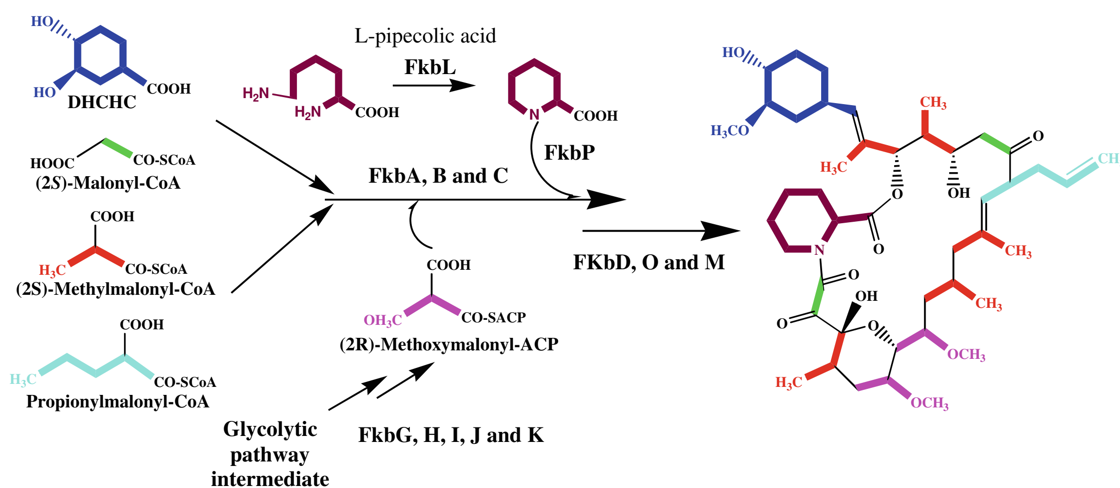

Fig. 1 The proposed biosynthetic pathway for FK506 (tacrolimus) FkbA, B, and C use a dihydroxycyclohexanecarboxylic acid (*DHCHC*) as a starter unit, and malonyl-CoA, methylmalonyl-CoA, propionylmalonyl-CoA and methoxylmalonyl-ACP as extender units. The FkbP then incorporates the L-pipecolic acid unit, and the macrolide ring is closed. Post-polyketide synthases (PKS) enzymes (FkbD, O, and M) produce FK506

Luria–Bertani, SOB, and SOC liquid media were used to grow the *E. coli* [31]. Ampicillin (100 μg/ml), apramycin (50 μg/ml), chloramphenicol (25 μg/ml), kanamycin (50 μg/ml), and nalidixic acid (25 μg/ml), all from Sigma–Aldrich (St. Louis, MO), were added to the growth media as required. At the beginning of cultivation, oils, such as corn oil, soybean oil and methyl oleate from Sigma–Aldrich, were added to the R2YE medium at a concentration of 5 g/l. In addition, at the beginning of cultivation, methyl oleate was added at various concentrations to the R2YE medium to determine the optimal initial methyl oleate concentration for efficient production of FK506.

Analysis of growth and FK506 production

The level of cell growth was measured by collecting duplicate 50-ml samples of fermentation broth at 24-h intervals, starting 24 h after inoculation. The mycelia were collected on preweighed filter paper (Whatman filter paper no. 1; Whatman, Maidstone, UK) by vacuum filtration. The samples were washed twice with distilled water, and filters containing mycelia were dried at 65°C and weighed.

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 evaporated to dryness under reduced pressure and then dissolved in 0.2 ml methanol for HPLC analysis. A portion of the solution was subjected to HPLC analysis. Analytical HPLC was carried out using a Waters (Milford, MA) model 2690 separations module on a Supelco Discovery HS C_{18} column (Bellefonte, PA), which was maintained at 50°C. The linear elution gradient ranged from 20 to 100% MeCN (0.1% phosphoric acid), the flow rate was 1 ml/min, and detection was at 205 nm. An authentic FK506 standard (Sigma–Aldrich) was used to construct a calibration curve of FK506 by HPLC analysis. The level of FK506 production reported is the average of two series of duplicate separation cultivations and extractions.

Expression of the pathways in *S. clavuligerus* CKD1119 providing methylmalonyl-CoA

Plasmid extraction and manipulation was carried out using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and QIAquick Gel Extraction kit (Qiagen). The QIAquick PCR Purification kit (Qiagen) was used for PCR product cleanup. The genomic DNA was isolated using a Wizard Genomic DNA Purification kit (Promega, Madison, WI). The cells were harvested by centrifugation, washed twice with distilled water, and then used to extract the genomic DNA. The PCR reactions were conducted using the GC-Rich PCR system (Roche, Indianapolis, IN).

The genes encoding the enzyme involved in the MCM pathway (*mutAB*) [8, 38] were cloned into the pSET152 derivative containing an *ermE** promoter, to yield pYJ1122. The *mutA* gene (coding for the small subunit of MCM) was obtained as a *Pac*I–*Xba*I PCR fragment from *S. erythraea* NRRL2338 genomic DNA and inserted into Litmus28. The oligonucleotide primer set used to amplify *mutA* was 5′-TTAATTAA ACTAGTCTGTCCGGCGCGACAAGCGTC-3′ (forward) and 5′-TCTAGACACCTCGAGCGAGCCGGCCAG-3′ (reverse) containing the *Pac*I, *Spe*I, *Xba*I, and *Xho*I sites (underlined), respectively. The *mutB* (coding for the large subunit of MCM) was obtained as an *Xho*I–*Xba*I PCR fragment from the *S. erythraea* NRRL2338 genomic DNA and inserted into the same site of Litmus 28 containing the *mutA* gene. The primer set used to amplify *mutB* was 5′-CTGGCCGGCTCGCTCGAGGTG-3′ (forward) and 5′-TCTAGAGGTCAGCCCGCGCTCGACG-3′ (reverse) containing the *Xho*I and *Xba*I sites (underlined), respectively. The *Pac*I–*Xba*I fragment of the *mutA* and *mutB* genes were obtained from the resulting Litmus 28, and ligated to the *Pac*I–*Xba*I sites of the pSET152 derivative containing an *ermE** promoter, yielding pYJ1122.

The genes encoding the enzyme in the malonyl/methylmalonyl-CoA ligase (*matB*) and methylmalonyl-CoA epimerase pathway [2, 23] were cloned into the pSET152 derivative containing an *ermE** promoter, yielding pYJ270. The *matB* and epimerase genes were obtained as *Bgl*II–*Pst*I and *Pst*I–*Xba*I PCR fragments from the *S. coelicolor* A3(2) genomic DNA and inserted into Litmus 28. The oligonucleotide primer set used to amplify *matB* was 5′-TCTCGCGGATCCGGCGCGATCCGGCAGCCT-3′ (forward) and 5′-ACTCCCCTGCAGTCAGGCGCTCAGTCACGG-3′ (reverse) containing the *Bgl*II and *Pst*I sites (underlined), respectively. The PCR product was digested with *Bgl*II–*Pst*I and ligated into the same sites of Litmus 28. The oligonucleotide primer set used to amplify the epimerase gene was 5′-GAGGGTCTGCAGGAATGTACTGAGCGGTATCCC-3′ (forward) and 5′-ACCCTATCTAGACCTATGTACGAGGGTCAGTGC-3′ (reverse) containing the *Pst*I and *Xba*I sites (underlined), respectively. The PCR product was excised with *Pst*I–*Xba*I and transferred to the same sites of Litmus 28 containing the *matB* gene. The *Bgl*II–*Xba*I fragment of the *matB* and epimerase genes were obtained from the resulting Litmus 28 and ligated to the *Bam*HI–*Xba*I sites of the pSET152 derivative containing an *ermE** promoter, generating pYJ270.

The genes encoding the enzymes involved in the propionyl-CoA carboxylase (PCC) pathway (*accAI*, *pccB*) [30] and the propionyl-CoA synthetase gene (*prpE*) [9, 24] were cloned into plasmid pSET152 derivative containing an *ermE** promoter, yielding pYJ269. The *accA1* and *pccB*

genes were obtained as *Bgl*II–*Nsi*I and *Nsi*I–*Hin*dIII PCR fragments from the *S. coelicolor* A3(2) genomic DNA, and the *prpE* gene was obtained as the *Hin*dIII–*Xba*I PCR fragment from the *S. typhimurium* LT2 ATCC 29631 genomic DNA and inserted Litmus 28. The set of oligonucleotide primers used to amplify *accAI* was 5′-ACGTTCAGATCTTGACTGTTCCGAACAGGG-3′ (forward) and 5′-ATCGTGATGCATGTCGTCATCGTTCAGTCC-3′ (reverse) containing the *Bgl*II and *Nsi*I sites (underlined), respectively. The PCR product was digested with *Bgl*II–*Nsi*I and ligated into the same sites of Litmus 28. The oligonucleotide primer set used to amplify *pccB* was 5′-ATCTCGATGCATCATGCAACCCACCCTAGG-3′ (forward) and 5′-TGGTCAAAGCTTCTCCTTACAGGGGGATGT-3′ (reverse) containing the *Nsi*I and *Hin*dIII sites (underlined), respectively. The PCR product was digested with *Nsi*I–*Hin*dIII and ligated into the same sites of Litmus 28 containing the *accAI* gene. The oligonucleotide primer set used to amplify *prpE* was 5′-CCAGCAAAGCTTCATTCTGGATGTCTCCCTGGA-3′ (forward) and 5′-CCACTATCTAGACGCTGAGTCTAACCCGTT-3′ (reverse) containing the *Hin*dIII and *Xba*I sites (underlined), respectively. The PCR product was digested with *Hin*dIII–*Xba*I and ligated into the same site of Litmus 28 containing the *accAI* and *pccB* genes. Finally, the *Bgl*II–*Xba*I fragment carrying the *accAI*, *pccB*, and *prpE* genes was obtained from the resulting Litmus 28 and placed into the *Bam*HI–*Xba*I sites of pSET152 derivative containing the *ermE** promoter, generating pYJ269.

pYJ1122, pYJ270. and pYJ269 were introduced into *S. clavuligerus* CKD1119 by conjugation from *E. coli* ET12567/pUZ8002, and the exconjugants were selected in the presence of 50 mg apramycin ml^{-1} using the standard protocol [12]. The level of FK506 production by the strains CKD1119/pYJ1122, CKD1119/pYJ270, and CKD1119/pYJ269 were determined in the fermentation procedure described above.

Analysis of intracellular acyl-CoA profile

For analysis of the intracellular acyl-CoA pools, trichloroacetic acid (TCA), CoA, and acetyl-, malonyl-, methylmalonyl-, succinyl-, and propionyl-CoA were purchased from Sigma–Aldrich. Silicone oils AR200 and DC200 were acquired from Fluka Chemie (Buchs, Switzerland), and the SPE (solid phase extraction) cartridge (OASIS HLB 3 cc/60 mg) and vacuum manifold were obtained from Waters. Ethylmalonyl-CoA was synthesized using a modification of the methods reported by Taoka et al. [26, 36]. All other reagents were of the purest grade commercially available.

Streptomyces clavuligerus CKD 1119 or *S. clavuligerus* CKD 1119/pYJ1122 was grown in R2YE medium at 28°C. On day 6, the mycelia were collected on filter paper by vacuum filtration, washed twice with distilled water, and transferred to a preweighed centrifuge tube to measure the wet weight biomass. When methyl oleate (10 m*M*) was added to the R2YE medium, *S. clavuligerus* CKD 1119 mycelia were collected on day 3 and prepared to measure the wet weight biomass as described above. At each examination, the wet cell weight was measured, and the weight of the cell was adjusted to equalize the amount of cells. Preparation of the mycelia extracts and analysis of the intracellular acyl-CoAs were performed using a modification of the methods reported by Park et al. [26]. The experiments were carried out in two duplicate series.

Result and discussion

Effect of methyl oleate on FK506 production

Oil is one of the carbon sources commonly used in fermentation medium to improve the level of polyketide production [16, 22, 27, 28]. The effects of various oils, including corn oil, soybean oil and methyl oleate, on cell growth and FK506 production were examined by adding each oil to the R2YE medium at a concentration of 5 g/l. Among the oils tested, the maximum level of FK506 production, approximately 11 mg/l, was obtained in the R2YE medium containing methyl oleate (data not shown). The concentration of methyl oleate added to the medium was varied (2.5, 5, 10, 20, 50 mM) to determine the optimum initial methyl oleate concentration for the production of FK506. The results are shown in Fig. 2a. The highest level of FK506 production, 15 mg/l, was obtained with an initial methyl oleate concentration of 10 m*M* (4.37 g/l); 50 m*M* methyl oleate reduced the level of FK506 production in the *S. clavuligerus* CKD1119 strain by approximately 64%.

The growth of *S. clavuligerus* CKD1119 and FK506 production were monitored over a 6-day incubation period with and without 10 m*M* methyl oleate supplementation. When methyl oleate was added, the levels of cell growth and FK506 production were approximately 1.7-fold and 2.5-fold higher, respectively, than that of the unsupplemented fermentations (Fig. 2b). In both cases, growth reached the stationary phase after 5 days. The maximum levels of FK506 production were observed on day 3 when methyl oleate was added to the medium and on day 6 without methyl oleate (Fig. 2b). In addition, methyl oleate increased the specific production of FK506 per cell weight by approximately 1.8-fold compared to that under the non-supplemented conditions (Fig. 2b). This result suggests that methyl oleate promotes both cell growth and FK506 production. However, a higher cell density is not the sole or major contributor to the increased production of FK506.

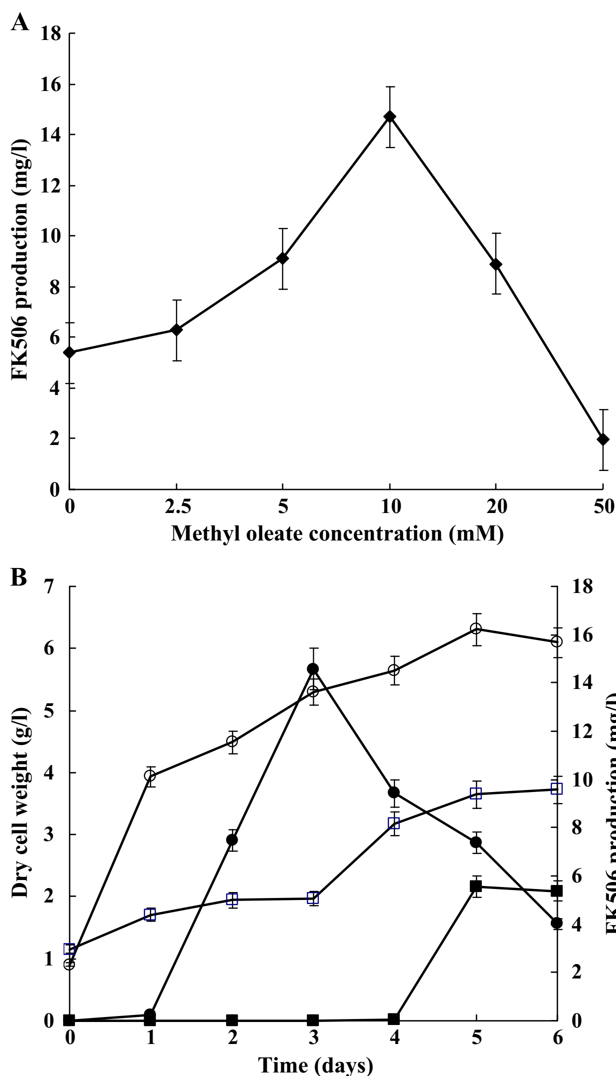

Fig. 2 Effect of methyl oleate on the production of FK506 by *S. clavuligerus* CKD 1119 in R2YE medium. **a** Effect of the methyl oleate concentration on FK506 titer in *S. clavuligerus* CKD 1119 fermentation. **b** Time course for cell growth and FK506 production in R2YE medium with and without 10 m*M* methyl oleate. *Solid square* FK506 concentration in R2YE medium without methyl oleate, *solid circle* FK506 concentration in R2YE medium with methyl oleate, *open square* cell growth FK506 concentration in R2YE medium without methyl oleate, *open circle* cell growth in R2YE medium with methyl oleate

Analysis of intracellular acyl-CoAs in *S. clavuligerus* CKD1119

It has been reported that ^{14}C-oleate is incorporated into FK506 in *S. clavuligerus* CKD1119 [13], which suggests that exogenous methyl oleate provides the building blocks for FK506 biosynthesis. Intracellular CoA-esters derived from *S. clavuligerus* CKD1119 grown with and without methyl oleate were examined to determine if the elevated production of FK506 by methyl oleate correlated with the differences in their in vivo CoA-ester composition, such as the high level of malonyl-CoA or methylmalonyl-CoA, both of which serve as precursors for FK506 biosynthesis. Due to the difference in growth rate and production time, the *S. clavuligerus* CKD1119 strain supplemented with methyl oleate was examined on day 3, and the unsupplemented strain was examined on day 6, when FK506 production had reached its maximum level under each condition. Significant differences in the levels of intracellular acyl-CoAs were observed in the two different media (Table 1). When the level of FK506 production reached its maximum, the level of methylmalonyl-CoA was 12.5-fold higher in the *S. clavuligerus* CKD1119 grown with methyl oleate than in the unsupplemented strain (Table 1), and the FK506 titers had increased by 2.5-fold (Fig. 2b). This result indicates that methyl oleate plays a significant role in providing methylmalonyl-CoA for FK506 biosynthesis. Another remarkable observation is that methyl oleate increased the levels of acetyl-CoA and succinyl-CoA by approximately 26.9-fold and 3.4-fold, respectively, over the non-supplemented strain. Interestingly, the intracellular malonyl-CoA level was reduced by methyl oleate supplementation. It is likely that more malonyl-CoA is exhausted during *S. clavuligerus* CKD1119 growth in methyl oleate-supplemented medium to produce more FK506.

The addition of methyl oleate to *Streptomyces hygroscopicus* NRRL B-1865 increased the level of polyether antibiotic production by 20-fold [6, 22], presumably by altering the membrane permeability and stimulating the uptake of valine, which is a known precursor of polyether and macrolide polyketides [3]. However, the feeding

Table 1 Intracellular acyl-CoAs profile in *Streptomyces clavuligerus* CKD1119 cultured in methyl oleate-supplemented and non-supplemented R2YE media at the maximum levels of FK506 production

Culture medium	CoA	Ac-	Ma-	Pr-	Su-	Me-	Et-	Total
R2YE	46 ± 6	29 ± 4	77 ± 8	99 ± 6	36 ± 4	121 ± 14	53 ± 3	461 ± 45
R2YE + methyl oleate	955 ± 37	782 ± 38	45 ± 5	85 ± 6	125 ± 12	1,521 ± 48	48 ± 6	3561 ± 152

Ac-, Et-, Ma-, Me-, Pr- and Su-, acetyl-, ethylmalonyl-, malonyl-, methylmalonyl-, propionyl-, and succinyl-CoA, respectively

All the values are corrected for recovery and are reported as pmoles/g cell weight ± standard deviation ($n = 2$)

studies carried out using valine or isobutyrate (a catabolite of valine) showed these two substances had no effect on the FK506 titers (data not shown). In another FK506-producer, *Streptomyces* sp. MA6858 (ATCC 55098) and the rapamycin (structurally similar to FK506)-producing *S. hygroscopicus* strain C9, valine had no effect on the production of polyketides [4, 40]. These results together with the incorporation of labeled oleic acid into FK506 in *S. clavuligerus* CKD1119 suggest that acetyl-CoA generated from methyl oleate degradation is metabolized into succinyl-CoA by the glyoxylate or citric acid cycles and that succinyl-CoA is then converted to methylmalonyl-CoA (Fig. 3), which may be a limiting precursor for FK506 biosynthesis. This hypothesis is consistent with the model predicted in the erythromycin-producing *S. erythraea* [27, 28], where an increased methylmalonly-CoA pool by the methylmalonyl-CoA mutase (MCM) pathway from succinyl-CoA enhances erythromycin production under oil-based growth conditions.

Identification of the effective metabolic pathway of methylmalonyl-CoA supply supporting FK506 biosynthesis in *S. clavuligerus* CKD1119

Four metabolic pathways can be considered for methylmalonyl-CoA supporting polyketide formation (Fig. 3): (1) isomerization of succinyl-CoA by the MCM [10, 27, 28, 37]; (2) carboxylation of propionyl-CoA via a reaction of propionyl-CoA carboxylase (PCC) [2, 24, 30]; (3) a multistep oxidation of isobutyryl-CoA generated from valine catabolism [29, 35]; and (4) the malonyl/methylmalonyl-CoA ligase (MatB) pathway [23]. Many studies have evaluated the role of these precursor feeding pathways in providing methylmalonyl-CoA for polyketide biosynthesis, and the general consensus is that the relative contributions of the different pathways are dependent on the strain and fermentation conditions. A knockout of the MCM gene, *mutB*, in erythromycin producers *S. erythraea* increased the level of erythromycin production in carbohydrate-based fermentation [27]. In oil-based fermentation, the same knockout in *mutB* has been found to reduce erythromycin production, and duplication of the MCM operon led to an increase in erythromycin production [27, 28]. Therefore, in *S. erythraea*, MCM acts to fill the methylmalonyl-CoA pool under oil-based fermentation conditions, while MCM acts in the reverse direction to decrease the methylmalonyl-CoA pool in carbohydrate-based fermentation. In monensin-producing *S. cinnamonensis*, the MCM pathway does not appear to contribute significantly to providing methylmalonyl-CoA for monensin production [5, 38, 42], in contrast to *S. erythraea*. Moreover, the increase in the monensin titers through oil-supplementation is probably associated with another pathway utilizing crotonyl-CoA reductase [3, 16].

We investigated the effect of the above-mentioned three pathways on the methylmalonyl-CoA level and FK506 production by heterologous expression of the MCM pathway (*S. erythraea mutAB* gene), MatB pathway [*matB* gene from *S. coelicolor* A3(2)], and the PCC pathway [*accA1* and *pccB* from *S. coelicolor* A3(2) and *prpE* from *S. typhimurium*) in *S. clavuligerus* CKD1119 (Fig. 4a). These heterologous genes were chosen based on the previously published examples of their successful application to

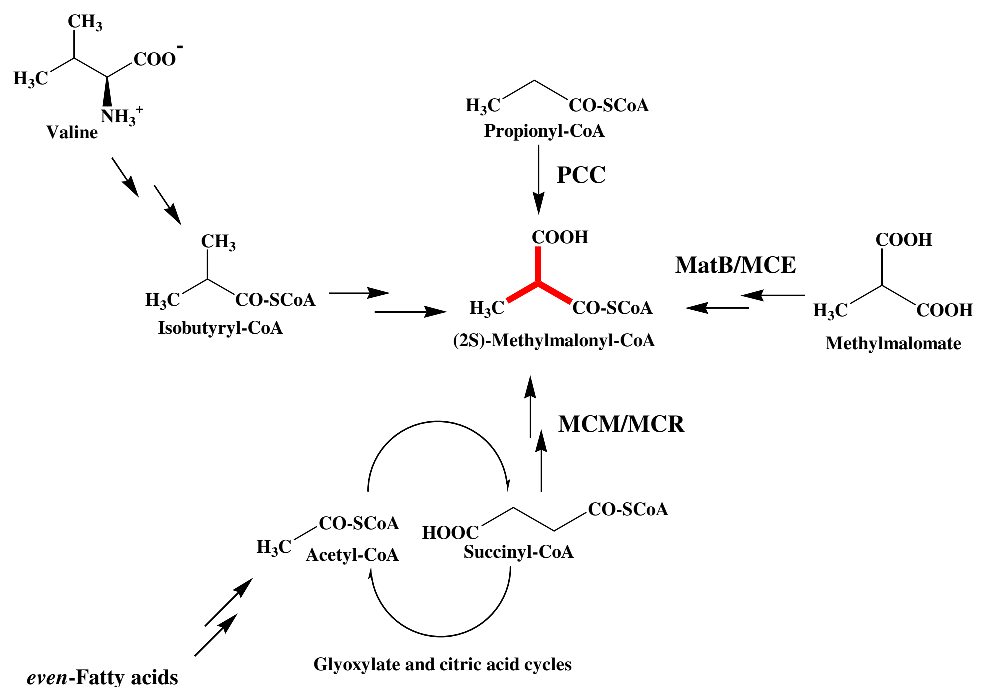

Fig. 3 The pathways for methylmalonyl-CoA formation. *PCC* Propionyl-CoA carboxylase, *MCM* methylmalonyl-CoA mutase, *MCR* methylmalonyl-CoA racemase, *MatB* malonyl/methylmalonyl-CoA ligase, *MCE* methylmalonyl-CoA epimerase

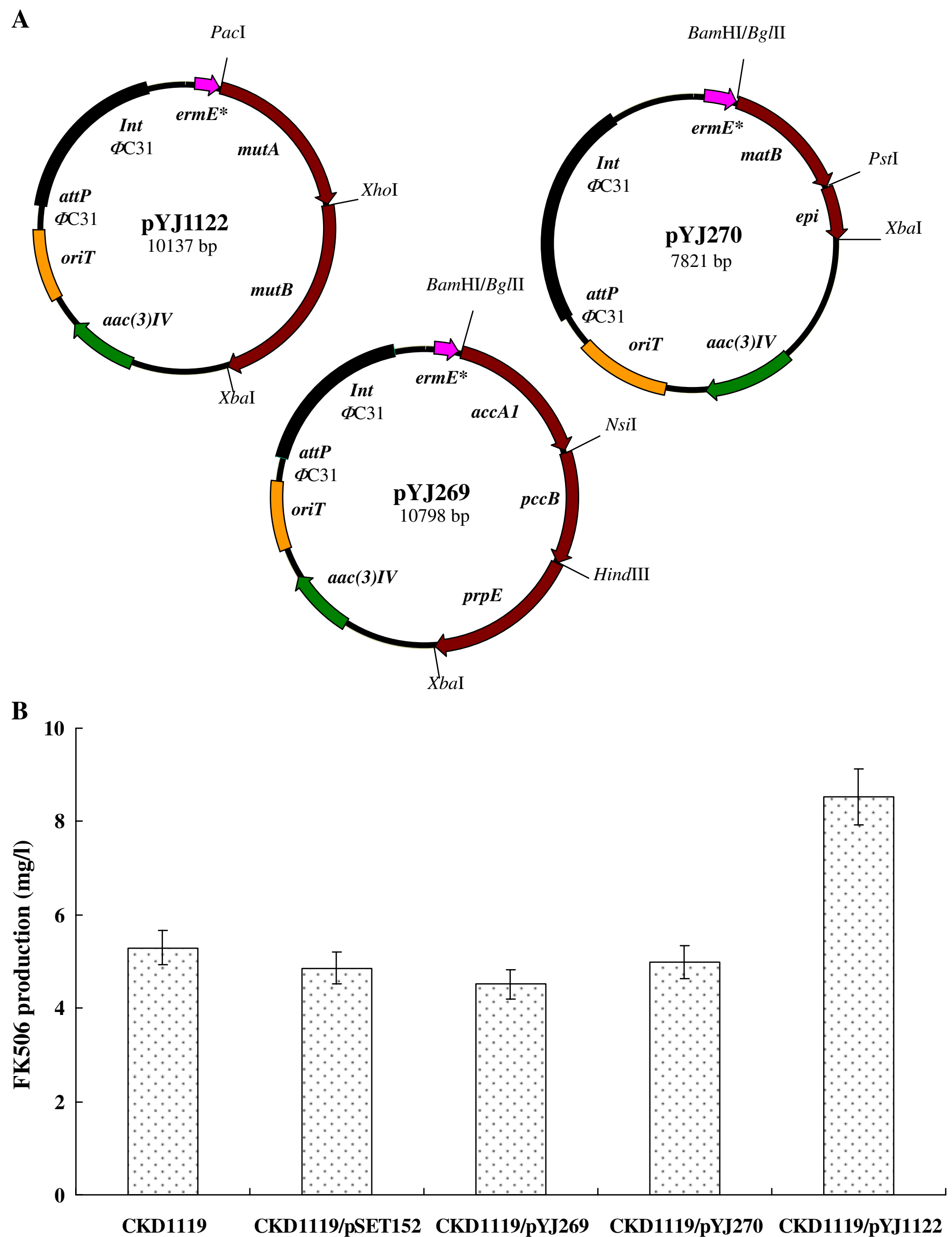

Fig. 4 The plasmids map for the methylmalonyl-CoA-providing pathway and FK506 production by heterologous expression of the methylmalonyl-CoA-providing pathways. **a** Plasmids for methylmalonyl-CoA providing were constructed into the pSET152 derivative containing an *ermE** promoter. The plasmids included a *Φ*C31-*attP* site for genomic integration (*thick black line*) and an apramycin-resistance marker [*aac*(*3*)*IV*]. **b** FK506 production by three heterologous expression of the MCM pathway (pYJ1122), MatB pathway (pYJ270), and PCC pathway (pYJ269). The values are the averages of two series of duplicate experiments

increasing the intracellular concentration of methylmalonyl-CoA in other microorganisms [23, 24, 28]. The valine catabolic pathway was excluded because valine or isobutyrate did not affect the FK506 titers in our study. Of the three pathways examined, only MCM led to a clear increase in the levels of FK506 and methylmalonyl-CoA (Fig. 4b). The addition of propionate or methyl malonate did not affect the level of FK506 production (data not shown), which is consistent with the observation that neither the PCC nor MatB pathways increased the production FK506 in *S. clavuligerus* CKD1119. The heterologous expression of *mutAB* genes in *S. clavuligerus* CKD1119 gave rise to a threefold increase in the methylmalonyl-CoA level and an approximately 3.5-fold decrease in the level of succinyl-CoA, compared to the levels present in the wild strain (Fig. 5a). The level of FK506 production in *S. clavuligerus* CKD1119 expressing the MCM pathway (*S. clavuligerus* CKD1119/pYJ1122) was also increased 1.5-fold as compared with *S. clavuligerus* CKD1119 (Fig. 5b). Furthermore, the addition of methyl oleate in the culture of *S. clavuligerus* CKD1119/pYJ1122 resulted in an additional twofold improvement in the FK506 titer as compared with *S. clavuligerus* CKD1119/pYJ1122 (3.4-fold compared to that observed in wild-type *S. clavuligerus* CKD1119 without methyl oleate) (Fig. 5b). This result supports the hypothesis that exogenous methyl oleate is

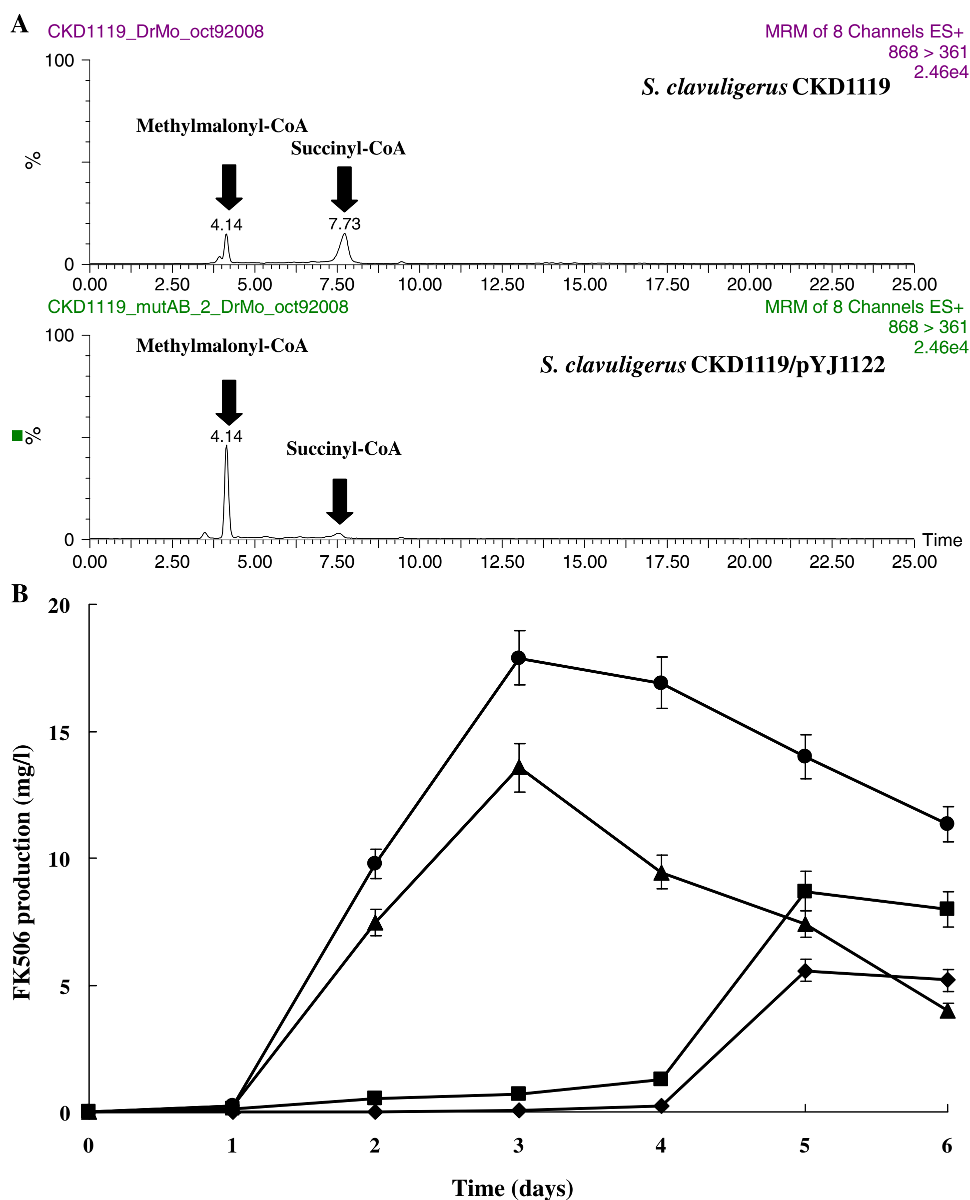

Fig. 5 Effect of the *mutAB* gene expression in *S. clavuligerus* CKD 1119. **a** Change in the profiles of methylmalonyl-CoA and succinyl-CoA by *mutAB* gene expression in *S. clavuligerus* CKD 1119. The chromatogram represents the multiple reaction mode (*MRM*) mass chromatogram of methylmalonyl- and succinyl-CoA (*m/z* 868 > 361). **b** Time course for FK506 production by both the expression of *mutAB* and the addition of exogenous 10 m*M* methyl oleate. *Solid diamond* CKD1119 without methyl oleate, *solid square* CKD1119/pYJ1122 without methyl oleate, *solid triangle* CKD 1119 with methyl oleate, *solid circle* CKD1119/pYJ1122 with methyl oleate. The values are averages of two series of duplicate experiments

catabolized to acetyl-CoA by β-oxidation, that acetyl-CoA generates succinyl-CoA, and that the MCM pathway plays a key role in increasing the methylmalonyl-CoA pool for FK506 biosynthesis in *S. clavuligerus* CKD1119. However, the addition of sodium succinate and hydroxocobalamin (necessary for MCM activity) at various concentrations did not affect the FK506 titers (data not shown), suggesting that the exogenously added succinate was not converted into FK506 and that hydroxocobalamin is not the limiting factor for methylmalonyl-CoA mutase activity in the MCM pathway in *S. clavuligerus* CKD1119 grown in the R2YE medium.

In this study, the level of FK506 production was increased by engineering the level of methylmalonyl-CoA in *S. clavuligerus* CKD1119 through a combination of chemical supplementation and genetic approaches. Although methylmalonyl-CoA has been suggested to be a limiting factor in the production of several polyketides, such as monensin, erythromycin, and FK506, the major methylmalonyl-CoA feeding pathway that can support high levels of production depends on the strain and growth conditions. These results suggest that among the different pathways examined in *S. clavuligerus* CKD1119, the MCM pathway is the dominant route for supplying methylmalonyl-CoA for the production of FK506. Although the results presented here are for the wild-type strain, the application of the same strain improvement mechanism to higher producing industrial strains may allow the development of more improved strains, provided that the supply of methylmalonyl-CoA is a limiting factor in those strains.

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