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Improvement of FK506 Production in *Streptomyces tsukubaensis* by Genetic Enhancement of the Supply of Unusual Polyketide Extender Units via Utilization of Two Distinct Site-Specific Recombination Systems

Dandan Chen,[a,b] Qi Zhang,[b] Qinglin Zhang,[b] Peilin Cen,[a] Zhinan Xu,[a] and Wen Liu[b]

Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou, China,[a] and State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, China[b]

FK506 is a potent immunosuppressant that has a wide range of clinical applications. Its 23-member macrocyclic scaffold, mainly with a polyketide origin, features two methoxy groups at C-13 and C-15 and one allyl side chain at C-21, due to the region-specific incorporation of two unusual extender units derived from methoxymalonyl-acyl carrier protein (ACP) and allylmalonyl-coenzyme A (CoA), respectively. Whether their intracellular formations can be a bottleneck for FK506 production remains elusive. In this study, we report the improvement of FK506 yield in the producing strain *Streptomyces tsukubaensis* by the duplication of two sets of pathway-specific genes individually encoding the biosyntheses of these two extender units, thereby providing a promising approach to generate high-FK506-producing strains via genetic manipulation. Taking advantage of the fact that *S. tsukubaensis* is amenable to two actinophage (ΦC31 and VWB) integrase-mediated recombination systems, we genetically enhanced the biosyntheses of methoxymalonyl-ACP and allylmalonyl-CoA, as indicated by transcriptional analysis. Together with the optimization of glucose supplementation, the maximal FK506 titer eventually increased by approximately 150% in comparison with that of the original strain. The strategy of engineering the biosynthesis of unusual extender units described here may be applicable to improving the production of other polyketide or nonribosomal peptide natural products that contain pathway-specific building blocks.

FK506 (tacrolimus), isolated from a variety of soil *Streptomyces* species, is a natural product with potent immunosuppressive activity. FK506 interacts with a receptor known as FK506-binding protein 12 (FKBP12); the FK506-FKBP12 complex then acts on a target protein, calcineurin, by inhibiting its Ser/Thr phosphatase activity. This leads to the arrest of T cell proliferation at the G_0-G_1 stage (18). As a consequence, FK506 has been clinically approved as an immunosuppressant to prevent the rejection of transplanted organs and for the treatment of various inflammatory diseases. Recently, other promising biological activities, such as neuroprotection and regeneration, were reported (9, 31), creating significant interest in the further development of FK506 for different medicinal uses.

FK506 biosynthesis involves a hybrid polyketide synthase (PKS)-nonribosomal peptide synthetase (NRPS) system, in line with the fact that the corresponding structure belongs to an amide bond-containing macrolide family with members that include its naturally occurring analogues FK520 and rapamycin (21, 22, 28, 35). FkbA to FkbC, constituting a typical type I PKS system, have functional domains that are organized colinearly with their activities in the biosynthetic assembly process: each module normally consists of ketoacylsynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) for chain elongation and optionally contains dehydratase (DH), enoylreductase (ER), and ketoreductase (KR) for reductively processing β-keto (oxidation state of beta-C in the growing polyketide chain) functionality. These PKSs are responsible for the construction of the main backbone of FK506 by catalyzing 10 two-carbon extensions from a chorismate-derived starter unit (2). An unnatural amino acid residue, L-pipecolate, terminates the resulting polyketide intermediate with an amide bond (7), whose formation is catalyzed by the NRPS FkbP, which subsequently closes the 23-member macrocyclic core via intramolecular lactonization (8). Besides malonyl-coenzyme A (M-CoA) and methylmalonyl (MM)-CoA, which often serve as common substrates of typical type I PKSs for elongation, the PKSs in FK506 biosynthesis feature the incorporation of two unusual extender units derived from methoxymalonyl (MOM)-ACP and allylmalonyl (AM)-CoA into the polyketide skeleton to produce two methoxy groups at C-13 and C-15 and one allyl side chain at C-21, respectively (Fig. 1A).

The entire biosynthetic gene cluster of FK506 has recently been characterized from several FK506-producing streptomycete strains (20), which shows that the formation of the unusual building blocks MOM-ACP and AM-CoA requires two sets of independent, pathway-specific genes (Fig. 1B and C). The first five contiguous genes, *fkbGHIJK*, with an organization identical to that of the FK520 biosynthetic gene cluster, were proposed to encode MOM-ACP generation, a process that starts with the utilization of 1,3-biphosphoglycerate (1,3-BPG) as the substrate. FkbH, which has both AT and phosphatase activities, is responsible for loading glyceroyl onto FkbJ, a discrete ACP protein, to produce glyceroyl-

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Address correspondence to Zhinan Xu, znxu@zju.edu.cn, or Wen Liu, wliu@mail.sioc.ac.cn.
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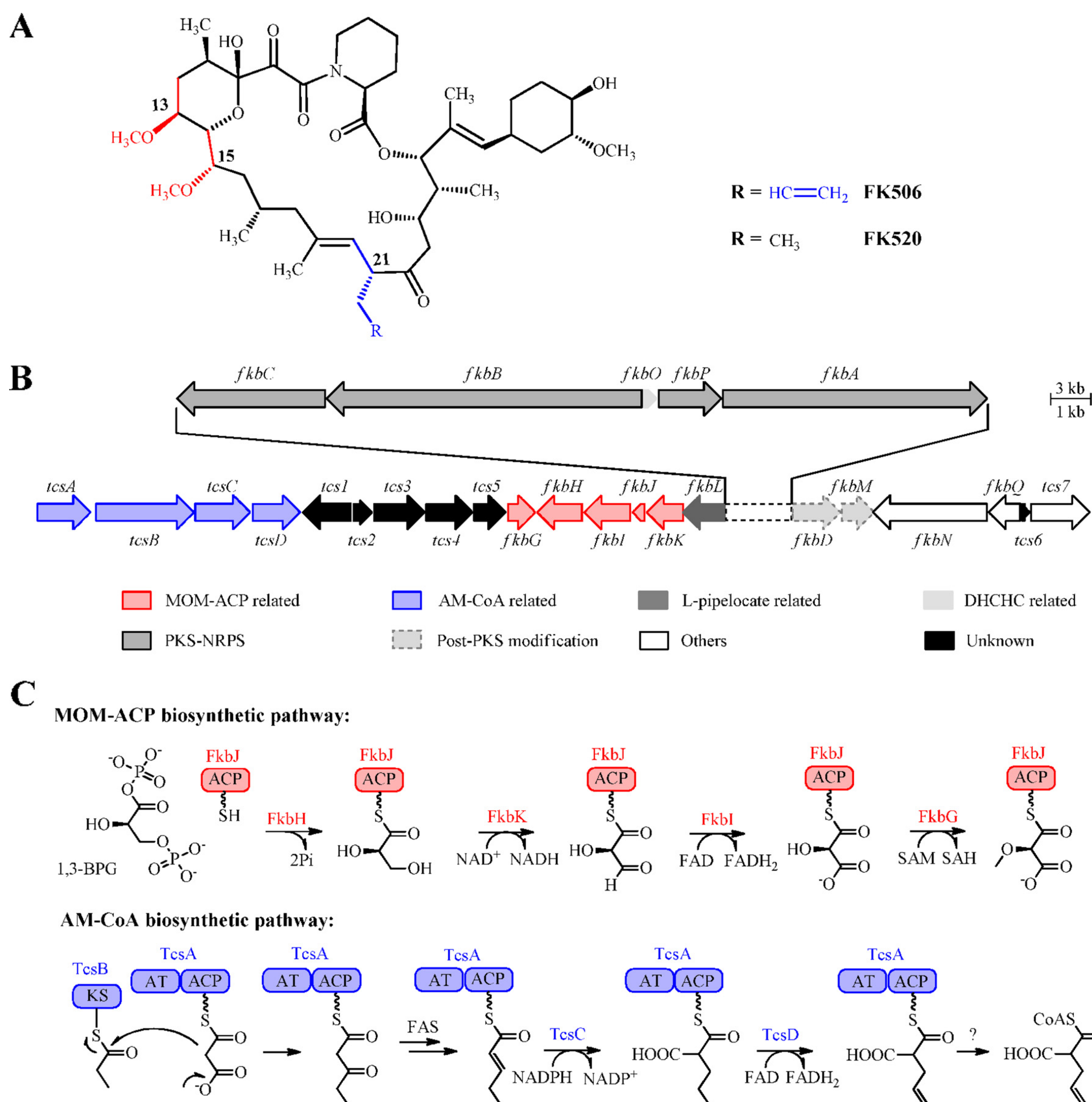

FIG 1 Chemical structures of FK506 and FK520, the FK506 biosynthetic gene cluster in *S. tsukubaensis*, and the biosynthetic pathways of MOM-ACP and AM-CoA. (A) Structures of FK506 and its analogue, FK520. The polyketide skeletons consist of two methoxy groups at C-13 and C-15, while the allyl side chain at C-21 of FK506 is replaced by an ethyl group in FK520. The FK506 macrocyclic scaffolds derived from MOM-ACP and AM-CoA are shown in red and blue, respectively. (B) Schematic representation of the FK506 biosynthetic gene cluster in *S. tsukubaensis*. Genes involved in the biosyntheses of MOM-ACP and AM-CoA are shown in red and blue, respectively. (C) Biosynthetic pathways of MOM-ACP and AM-CoA, catalyzed by FkbG-K and TcsA-D, respectively. DHCHC, which stands for (4R,5R)-4,5-dihydroxycyclohex-1-enecarboxylic acid, is the chorismate-derived starter unit.

ACP for sequential elaborate modifications by two dehydrogenases, FkbK and FkbI, and an *O*-methyltransferase, FkbG, to furnish the MOM-ACP unit (4, 14). In contrast, the second set of four contiguous genes, *tcsABCD*, identified from the FK506 biosynthetic gene cluster are unique, and more recently, their deduced products have been confirmed to form a noncanonical discrete PKS system to catalyze AM-CoA formation in coordination with the fatty acid synthase (FAS) pathway. Functioning as a priming KS, the propionyl-acylated TcsB catalyzes the condensation of the propyl moiety with malonate loaded on TcsA, an AT-ACP bidomain protein, to form an ACP-tethered β-keto-pentanoate, which may then be converted into *trans*-2-pentenyl-ACP by involving the KR and DH activities of FAS of the host. Further modifications of the pentenyl group, including reductive carboxylation by TcsC in a manner similar to that of crotonyl-CoA carboxylase/reductase (CCR) (17), as well as dehydrogenation by the FAD-dependent enzyme TcsD, form the ACP-tethered allylmalonate, which is likely released as AM-CoA, an unprecedented extender unit for the FK506 assembly PKS system to build the side chain as allyl (10, 20).

Recent progress in understanding the biosyntheses of MOM-ACP and AM-CoA opens the door to improve FK506 production by intracellular enrichment of the precursor supply. To achieve this, genetic manipulations have frequently been used, including phage integrase-mediated recombination. The integrase recognizes the bacterial *attB* site, where a conservative and reciprocal

TABLE 1 Bacterial strains and plasmids used and constructed in this study

Strains/plasmids	Characteristic(s)	Source/reference
E. coli		
DH5α	Host for general cloning	Invitrogen
ET12567(pUZ8002)	Donor strain for conjugation between *E. coli* and *Streptomyces*	15
BW25113 (pIJ790)	Host for Red/ET-mediated PCR-targeted mutagenesis	11
S. tsukubaensis		
ZJU01	Original FK506-producing strain	CGMCC[a] 51771
FL101	Derivative of ZJU01 containing pSET152 integrated by the ΦC31 integrase-mediated recombination	This study
FL102	Derivative of ZJU01 containing pSOK804 integrated by the VWB integrase-mediated recombination	This study
FL103	Derivative of ZJU01 containing pFL103 integrated by the ΦC31 integrase-mediated recombination with the genotype of *PermE*-fkbGKJIH*	This study
FL104	Derivative of ZJU01 containing pFL104 integrated by the ΦC31 integrase-mediated recombination with the genotype of *PermE*-tcsABCD*	This study
FL105	Derivative of FL101 containing pTA804 integrated by the VWB integrase-mediated recombination	This study
FL106	Derivative of FL103 containing pFL106 integrated by the VWB integrase-mediated recombination with the genotype of *PermE*-tcsABCD*	This study
FL107	Derivative of FL104 containing pFL107 integrated by the VWB integrase-mediated recombination with the genotype of *PermE*-fkbGKJIH*	This study
Plasmids		
pMD18-T simple	*E. coli* subcloning vector	TaKaRa
pSET152	*E. coli-Streptomyces* shuttle vector containing the *aac(3)IV* gene and the ΦC31 *attP* site and integrase gene	3
pSOK804	*E. coli-Streptomyces* shuttle vector containing the *aac(3)IV* gene and the VWB *attP* site and integrase gene	29
pWHM79	pGEM3zf derivative carrying a 0.5-kb fragment containing the *ermE** promoter	30
pTA804	pSOK804 derivative replacing the *aac(3)IV* gene with the *bla* and *tsr* genes	This study
pMD-G	pMD18-T derivative carrying *fkbG* without its terminator	This study
pMD-HIJK	pMD18-T derivative carrying *fkbHIJK* with its terminator	This study
pMOM	pMD18-T derivative carrying *fkbG-fkbK-fkbJ-fkbI-fkbH*	This study
pAM	pMD18-T derivative carrying *tcsA-tcsB-tcsC-tcsD*	This study
pFL103	pSET152 derivative for the *PermE**-controlled expression of *fkbG-fkbK-fkbJ-fkbI-fkbH*	This study
pFL104	pSET152 derivative for the *PermE**-controlled expression of *tcsA-tcsB-tcsC-tcsD*	This study
pFL106	pTA804 derivative for the *PermE**-controlled expression of *tcsA-tcsB-tcsC-tcsD*	This study
pFL107	pTA804 derivative for the *PermE**-controlled expression of *fkbG-fkbK-fkbJ-fkbI-fkbH*	This study

[a] CGMCC, China General Microbiological Culture Collection.

exchange (involving a cleavage and subsequent recombination process) takes place with the phage *attP* site of the introduced DNA to form the hybrid sequences *attL* and *attR* (6). In this study, we exploited the sequence information regarding the phage attachment sites in the FK506 producer *Streptomyces tsukubaensis* ZJU01, showing that two distinct site-specific recombinations, based on the ΦC31 and VWB integration systems, respectively, are operable and have no apparent effect on FK506 production. Based on this, we report the introduction of additional copies of the pathway-specific genes into *S. tsukubaensis* at the selected *attB* loci on the chromosome, aiming at improvement of FK506 production by genetic enhancement of the biosyntheses of the unusual building blocks MOM-ACP and AM-CoA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and reagents. The bacterial strains and plasmids used in this study are summarized in Table 1. The primer sequences are listed in Table 2. The biochemicals, chemicals, media, restriction enzymes, and other molecular biological reagents were purchased from standard commercial sources unless otherwise stated.

DNA isolation, manipulation, and sequencing. DNA isolation and manipulation in *Escherichia coli* and *S. tsukubaensis* were performed according to standard protocols (15). PCR amplifications were carried out on an Authorized Thermal TM Cycler (AG 22331; Eppendorf, Hamburg, Germany) using either *Taq* DNA polymerase or PrimeStar HS DNA polymerase (TaKaRa). Primer synthesis and DNA sequencing were performed at Shanghai GeneCore Biotechnology Inc.

Construction of pTA804 as a derivative of pSOK804. According to the previously described methods (11, 12), pTA804 was constructed via Red/ET-mediated recombination by substituting *bla* (ampicillin resistance gene) and *tsr* (thiostrepton resistance gene) for *aac(3)IV* (apramycin resistance gene). A gene cassette containing *bla* and *tsr* was amplified from plasmid pSET151 (3) using the primer pair p151Redf/p151Redr, which was further introduced into *E. coli* BW25113(pIJ790) containing pSOK804 (29) by electroporation. The positive clones were selected through their ampicillin-resistant phenotype and were sequenced to confirm the fidelity of the λ Red-dependent recombination.

Construction for duplication of pathway-specific genes. To duplicate the MOM-ACP pathway-specific genes *fkbGHIJK*, a 0.8-kb fragment containing *fkbG* without its native terminator and a 3.5-kb fragment containing *fkbHIJK* were amplified from the genomic DNA of ZJU01 by PCR amplification using the primer pairs pGf/pGr and pHIJKf/pHIJKr, respectively. Both PCR products were cloned into pMD18-T to yield pMD-G and pMD-HIJK. After sequencing to confirm fidelity, the 3.5-kb SpeI/XbaI fragment of pMD-HIJK was inserted into the XbaI site of pMD-G to generate pMOM, the genotype of which was validated by digestion analysis of restriction enzymes. The 4.3-kb HindIII/XbaI fragment of pMOM and a 0.5-kb EcoRI/HindIII fragment containing the constitutive *ermE** promoter (*PermE**) from pWHM79 (30) were then coligated into the EcoRI/XbaI site of pSET152 to generate pFL103.

To duplicate the AM-CoA pathway-specific genes *tcsABCD*, a 6.4-kb

TABLE 2 Primers used for gene cloning, genotype verification, and RT-PCR amplification in this study

Name	Sequence[a]	Description
p151Redf	gtgcaatacgaatggcgaaaagccgagctcatcggtcagTTACCAATGCTTAATCAGTG	Cloning of the *bla* and *tsr* genes from pSET151 for Red/ET-mediated recombination
p151Redr	tcagccaatcgactggcgagcggcatcgcattcttcgcaTTATCGGTTGGCCGCGAGAT	
pGf	TAAGCTTCGAGGCGCCGCTGGACCGTGTGG (HindIII)	Cloning of the *fkbG* gene without its terminator from ZJU01 genome
pGr	TTCTAGACGTTCACCGCTTCCGCAACAAGG (XbaI)	
pHIJKf	TACTAGTGGCAGCGCCTTCGCCGACCACATC (SpeI)	Cloning of the *fkbHIJK* genes with their terminator from ZJU01 genome
pHIJKr	TTCTAGACAATGCGGGGCTGCGCGACGACG (XbaI)	
pABCDf	TAAGCTTCCATCCCGATCATGCCCCTCCTG (HindIII)	Cloning of the *tcsABCD* genes with their terminator from ZJU01 genome
pABCDr	TTCTAGAAGGCCCTGACGCGGGACTGACC (XbaI)	
ATTB1	CGGGATCCGACCC(G/C)TTCATCATGATGGAC	Degenerate primer pair from reference 5 to identify the ΦC31 *attB* sites
ATTB2	TGGAATTCAGGTT(G/C)ACCCA(C/G)AGCTG(G/C)AG	
pL-C31f	CCCACAGCTGGAGGCCGTGG	Verification of the hybrid *attL* site from the ΦC31-based recombination
pL-C31r	CAGGGCGAGCAATTCCGAGA	
pR-C31f	CAGAGCAGGATTCCCGTTGAG	Verification of the hybrid *attR* site from the ΦC31-based recombination
pR-C31r	CCCTTCATCATGATGGACCAG	
pL-VWBf	GCCTTCGTAGCTCAGGGGATAGAG	Verification of the hybrid *attL* site from the VWB-based recombination
pL-VWBr	CGCCGCCGCCTTTTCCTCAATCG	
p152f	GTAACGCCAGGGTTTTCCCAGTCACG	Verification of pSET152-based gene duplication at the ΦC31 *attB* site
p152r	TCCGGCTCGTATGTTGTGTGGAATTG	
p804f	AGCAGGCGTTCCTCGGCGTCCTCGT	Verification of pTA804-based gene duplication at the VWB *attB* site
p804r	AGCTCACTCATTAGGCACCCCAGGC	
pMOMf	CTCCCAGTACGGCCTGCCCACCTCC	Verification of gene duplication for the MOM-ACP biosynthetic pathway
pMOMr	TGCGCTCCCTGGACCTGAGAATGACG	
pAMf	GAAGCGCTCCCGGGGCGCGGATATC	Verification of gene duplication for the AM-CoA biosynthetic pathway
pAMr	CAATGGGCTGCGGCTGCTGGAGATC	
pRTGf	CTGGGCGGTCTGCGGGCGGAGTCG	RT-PCR amplification of a 102-bp *fkbG* fragment representing the *fkbG* mRNA level
pRTGr	GGTCAACCGCACCAGGAATTCCAG	
pRTKf	GCGGACCTCATCGGCATCGACAAC	RT-PCR amplification of a 102-bp *fkbK* fragment representing the *fkbHIJK* mRNA level
pRTKr	CTTGGTGAGGAGCAGGTCGCAGGG	
pRTAf	GGCGTCTTCGCCGGTCTGGGTGCC	RT-PCR amplification of a 106-bp *tcsA* fragment representing the *tcsABCD* mRNA level
pRTAr	GAAGCGGCCGGACCGGCTTCAGCC	
pRTDf	GTGCCGGGGGACAACGCGCTGAAC	RT-PCR amplification of a 103-bp *tcsD* fragment representing the *tcsABCD* mRNA level
pRTDr	GGGCGACACCCAGAGCGGATGCGG	
pRTPf	CAGATGCTGCACTCGCACGGTTCG	RT-PCR amplification of a 102-bp *fkbP* fragment as a control
pRTPr	GTTGAGGGCGGGGTGGTCGAGCGG	

[a] Lowercase letters indicate the sequences for Red/ET-mediated recombination. Restriction enzyme sites are underlined.

fragment containing *tcsABCD* was amplified by PCR amplification using the primer pair pABCDf/pABCDr with the genomic DNA of ZJU01 as the template. This fragment was cloned into pMD18-T to yield pAM. After sequencing to confirm fidelity, the 6.4-kb HindIII/XbaI fragment of pAM and a 0.5-kb EcoRI/HindIII fragment containing *PermE** were coligated into the EcoRI/XbaI site of pSET152 to yield pFL104.

The pTA804 derivatives pFL106 and pFL107 were constructed similarly to pFL103 and pFL104 by inserting the corresponding DNA fragments into pTA804 instead of pSET152.

Generation of recombinant strains and confirmation of genotypes. The pSET152 and pSOK804 derivatives were introduced into *S. tsukubaensis* ZJU01 by intergeneric conjugation from *E. coli* ET12567(pUZ8002), following the procedure described previously (15).

For pSET152-based recombination (as for FL101, FL103, and FL104), colonies with an apramycin-resistant phenotype were identified as exconjugants, the genotypes of which were further confirmed by PCR amplification-coupled sequencing using the primer pairs pL-C31f/pL-C31r and pR-C31f/pR-C31r. For pSOK804-based recombination (as for FL102), colonies resistant to apramycin were identified as exconjugants, the genotypes of which were verified by PCR-based amplification and sequencing by using the primer pair pL-VWBf/pL-VWBr. For pSET152- and pTA804-based recombination (as for FL105, FL106, and FL107), colonies resistant to both apramycin and thiostrepton were identified as exconjugants, the genotypes of which were then verified by PCR amplification and DNA sequencing by using the primer pairs pL-C31f/pL-C31r, pR-C31f/pR-C31r, and pL-VWBf/pL-VWBr.

To distinguish individual recombinant strains, different combinations of primers corresponding to the sequences of the conjugative vector (pSET152 or pTA804) and the duplicated gene cassette (*fkbGHIJK* or *tcsABCD*) were used in PCR-based amplification and sequencing to identify the specificity of each genotype (see Fig. 3A).

FK506 production in *S. tsukubaensis*. *S. tsukubaensis* ZJU01 and its derivatives were grown on agar plates (with the appropriate antibiotic[s] for recombinant strains) with alanine-starch agar (AS-1) medium consisting of 0.1% yeast extract, 0.02% L-alanine, 0.05% L-arginine, 0.5% soluble starch, 0.25% NaCl, 1.0% Na_2SO_4, and 2.0% agar at pH 7.5 and 30°C for sporulation. For fermentation, an agar piece of approximately 1 cm^2 was inoculated into a 250-ml flask containing 25 ml of the seed medium, consisting of 1.0% (vol/vol) glycerol, 4.0% soybean meal, 1.0% soluble starch, and 0.2% $CaCO_3$ at pH 7.0 ± 0.2, and was maintained at 28°C and 220 rpm for 24 to 28 h. Five milliliters of the seed culture was inoculated into a 500-ml flask containing 50 ml of the fresh fermentation medium [2.0% soluble starch, 1.0% glucose, 1.0% (vol/vol) soybean oil, 0.1% L-lysine, 0.17% $(NH_4)_2SO_4$, 0.05% K_2HPO_4, 0.05% NaCl, 0.05% $MgSO_4 \cdot H_2O$, 0.5% $CaCO_3$, and trace elements as described previously (36), pH 7.0 ± 0.2]. The cultivations were maintained at 28°C and 220 rpm. For each strain, at least three independent isolates were subjected to parallel fermentation experiments in triplicate.

Chemical analysis of FK506 and FK520 production. Culture samples (2 ml) were centrifuged for 10 min at 12,000 rpm. The supernatant was extracted twice with 2 ml of ethyl acetate, and the mycelium was extracted with 2 ml of acetone. The organic extracts were combined, evaporated to dryness under reduced pressure, and redissolved in 0.1 ml of acetonitrile.

High-performance liquid chromatography (HPLC) analysis was car-

ried out on a Diamonsil C_{18} (2) 5μ column (250 × 4.6 mm; catalog no. 99603; Dikma Technologies) on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). An isocratic program (60% acetonitrile and 40% H_2O) was carried out at a flow rate of 1 ml/min, and the temperature was maintained at 60°C. Liquid chromatography-mass spectrometry (LC-MS) analysis was carried out with the same isocratic program on an LC-MS 2010 A liquid chromatograph-mass spectrometer (Shimadzu, Japan), showing $[M+Na]^+$ ions at *m/z* 826.5 and $[M+NH_4]^+$ ions at *m/z* 821.5, in accordance with the FK506 molecular formula $C_{44}H_{69}NO_{12}$, and $[M+Na]^+$ ions at *m/z* 814.5 and $[M+NH_4]^+$ ions at *m/z* 809.5, in accordance with the FK520 molecular formula $C_{43}H_{69}NO_{12}$, respectively. The FK506 and FK520 titers were quantified using UV detection at 215 nm and were calculated with standard curves, representing the average values of at least three series of three parallel fermentation experiments.

Transcriptional analysis. Each total-RNA sample was prepared from *S. tsukubaensis* mycelia after 36, 72, or 108 h of fermentation with TRIzol reagents (Invitrogen) according to the manufacturer's instructions. The DNase I-treated mRNA (5 ng) was reverse transcribed to the first-strand cDNA using the ReverTra Ace-α-kit (Toyobo). As an additional control for each primer set and RNA sample, the cDNA synthesis reaction was carried out in the absence of reverse transcriptase to verify that genomic DNA did not contaminate the RNA samples. After incubation with RNase H, the resultant cDNAs were used as templates for PCR to analyze the transcription levels of the MOM-ACP and/or AM-CoA biosynthetic genes. For each *S. tsukubaensis* strain, three independent isolates were subjected to RNA preparation for PCR amplification analysis in parallel.

The amplification of each sample was performed by using *Taq* DNA polymerase under the following conditions: for predenaturation, 2 min at 94°C, and for each cycle, 30 s at 94°C followed by 30 s at 65°C and 10 s at 72°C. A 102-bp fragment of *fkbG* and a 102-bp fragment of *fkbK* (representatives of MOM-ACP biosynthetic genes), as well as a 106-bp fragment of *tcsA* and a 103-bp fragment of *tcsD* (representatives of AM-CoA biosynthetic genes) were amplified using the primer pairs pRTGf/pRTGr, pRTKf/pRTKr, pRTAf/pRTAr, and pRTDf/pRTDr, respectively. A 102-bp fragment of the NRPS gene *fkbP* was amplified using the primer pair pRTPf/pRTPr and served as an internal control. The amplification of each gene was optimized by using the 72-hour sample from the original strain as the template, leading to determination of a common 30-cycle reaction that clearly shows the difference in mRNA abundance (data not shown).

RESULTS

Establishment of two actinophage (ΦC31 and VWB) integrase-mediated recombination systems in *S. tsukubaensis*. Genetic studies on the diversity of the phage ΦC31 *attB* sites from different *Streptomyces* species revealed a conservative region within an open reading frame (ORF) coding for a putative chromosome condensation protein (5). With the degenerate primer pair ATTB1/ATTB2 (5), we successfully amplified a 294-bp DNA fragment by PCR from the genomic DNA of ZJU01, which has the 51-bp conservative sequence for ΦC31 attachment (Fig. 2A). Based on this, a ΦC31-derived integrative vector, pSET152, was introduced into ZJU01 by conjugation to generate the recombinant strain FL101, with a frequency of 2×10^{-6} exconjugants per recipient spore, by using the methylation-deficient *E. coli* ET12567(pUZ8002) as the donor. To verify the anticipated integration, we randomly selected five apramycin-resistant exconjugants for template preparation. PCR amplification-coupled DNA sequencing clearly indicated that all of the exconjugants exhibited identical genotypes of $attL^{\Phi C31}$-linear pSET152-$attR^{\Phi C31}$ at the integrating locus (Fig. 2A and 3). Thus, the ΦC31-based system is applicable to site-specific recombination in *S. tsukubaensis*.

As demonstrated in *S. venezuelae* and *S. ghanaensis*, the *attB* site of actinophage VWB resides in a putative $tRNA^{Arg}$ (AGG) gene (23, 33), which is different from that of ΦC31. To verify the availability of the $attB^{VWB}$ site on the chromosome of ZJU01, we introduced a VWB-derived integrative vector, pSOK804, into ZJU01 by conjugation, yielding the recombinant strain FL102 at a frequency of 5×10^{-5} per recipient spore. By comparing the $attB^{VWB}$ regions from *S. venezuelae* and *S. ghanaensis*, we identified a specific 74-bp highly similar sequence, which is widespread in various *Streptomyces* genomes (Fig. 2B). The 74-bp sequence was observed to appear in the conjunctive $attL^{VWB}$ site after integration (33), which prompted us to select the conserved $attL^{VWB}$ site, resulting from the hybridization of the 5′-terminal sequence of $attB^{VWB}$ and the 3′-terminal sequence of $attP^{VWB}$ of pSOK804, to validate the genotype of FL102. We performed PCR-based amplification and sequencing on five randomly selected pSOK804-based exconjugants. As expected, all five samples contained identical 74-bp sequences within the hybrid $attL^{VWB}$ site, supporting the genotype of FL102 as $attL^{VWB}$-linear pSOK804-$attR^{VWB}$, which was produced by the site-specific integration of pSOK804 (Fig. 2B and 3). Therefore, *S. tsukubaensis* proved to be amenable to the VWB integrase-mediated recombination system.

Effect of site-specific recombination on FK506 production in *S. tsukubaensis*. The identification of two distinct *attB* sites on the chromosome of ZJU01 provides us a choice of the utilization of two different site-specific recombination systems in the insertion of an artificial gene construct. To apply this approach, we evaluated the effects of the ΦC31- and VWB-based integrations on *S. tsukubaensis*. pSET152-based integration at the $attB^{\Phi C31}$ locus did not affect the growth characteristics, and the resulting strain, FL101 (43.4 ± 4.1 mg/liter), produced FK506 in a quantity similar to that of the original strain, ZJU01 (46.9 ± 2.5 mg/liter). In a similar manner, FL102, which bears an insertion of pSOK804 at the $attB^{VWB}$ locus, also showed no apparent difference in morphology and in FK506 production (40.7 ± 6.7 mg/liter) (Fig. 3C).

Duplication of the *fkbGHIJK* cassette encoding MOM-ACP formation via the ΦC31-based recombination system. FkbG and FkbH to FkbK are convergently transcribed on the *S. tsukubaensis* chromosome (Fig. 1B). To simplify plasmid construction, *fkbG* without its native terminator was amplified and placed upstream of *fkbHIJK*, and the resultant fragment was inserted into pSET152 under the control of the constitutive *ermE** promoter (*PermE**) to give pFL103. pFL103 was introduced into ZJU01 by conjugation to yield the recombinant strain FL103, the genotype of which was verified by PCR amplification-coupled sequencing as $attL^{\Phi C31}$-linear pFL103-$attR^{\Phi C31}$ (Fig. 3A and B). The highest FK506 titer of FL103 was observed after 6 days of fermentation, similar to that of the wild-type strain ZJU01 (Fig. 3C). FL103 produced FK506 with a maximal titer of 61.3 ± 2.3 mg/liter under the specified culture conditions, showing an approximate 30% improvement in comparison with ZJU01 (46.9 ± 2.5 mg/liter). The data indicated that the duplication of the *fkbGHIJK* cassette has a positive effect on FK506 production.

Duplication of the *tcsABCD* cassette encoding AM-CoA formation via the ΦC31-based recombination system. pFL104, a pSET152-derived plasmid containing the AM-CoA pathway-specific genes *tcsABCD* under the control of *PermE**, was introduced into ZJU01 to yield the recombinant strain FL104, the genotype of which was confirmed as $attL^{\Phi C31}$-linear pFL104-$attR^{\Phi C31}$ (Fig. 3A and B). Similarly, FK506 production by FL104 reached the maximum after 6 days of fermentation (Fig. 3C), with a yield of 95.7 ±

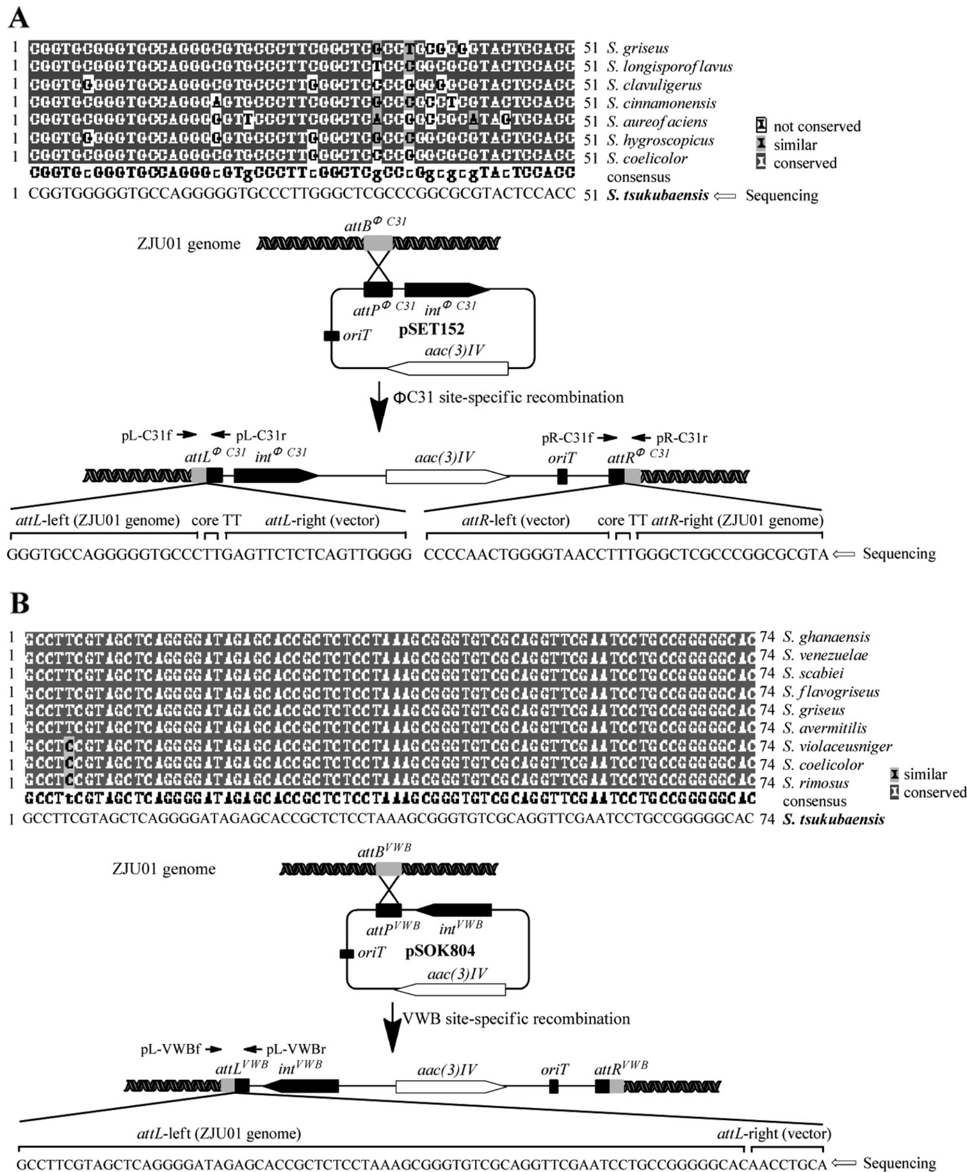

FIG 2 Identification and confirmation of the ΦC31 and VWB *attB* sites on the chromosome of *S. tsukubaensis*. (A) Nucleotide sequence alignment revealed a 51-bp conserved region within the $attB^{\Phi C31}$ locus in various streptomycete species. PCR amplification-coupled DNA sequencing confirmed the existence of the $attB^{\Phi C31}$ locus in *S. tsukubaensis* and the regions surrounding the hybrid $attL^{\Phi C31}$ and $attR^{\Phi C31}$ after the site-specific integration of pSET152. (B) Nucleotide sequence alignment revealed a 74-bp highly conserved region widespread in various streptomycete genomes. Sequence analysis of the PCR products revealed the region surrounding the hybrid $attL^{VWB}$ after site-specific integration of pSOK804.

4.6 mg/liter, approximately 100% above that of ZJU01. The duplication of the *tcsABCD* cassette significantly improved the FK506 fermentation yield, suggesting that AM-CoA formation in cells can be one of the main rate-limiting factors for FK506 biosynthesis in *S. tsukubaensis*.

Coduplication of the *tcsABCD* and *fkbGHIJK* cassettes via both the ΦC31- and VWB-based recombination systems. Inspired by the success of FL103 (duplicating *fkbGHIJK* alone) and FL104 (duplicating *tcsABCD* alone) in FK506 production at an apparently improved level, we investigated whether the enhancement of both the MOM-ACP and AM-CoA pathways would result in a greater degree of production improvement. We thus developed a strategy in which both the ΦC31- and VWB-based systems could be utilized simultaneously. Since *S. tsukubaensis* is sensitive to apramycin and thiostrepton, we constructed a new VWB-based shuttle plasmid as a complement of pSET152. By replacing the original apramycin resistance gene on pSOK804 with the ampicillin and thiostrepton resistance genes, we generated a new conjugative vector, pTA804. We obtained FL105, a derivative of FL101, by inserting linear pTA804 at the $attB^{VWB}$ locus by conjugation and thiostrepton resistance selection. This recombinant strain, with an expected genotype of $attL^{\Phi C31}$-linear pSET152-$attR^{\Phi C31}$-$attL^{VWB}$-linear pTA804-$attR^{VWB}$, shown in Fig. 3A, produced FK506 (40.1 ± 4.5 mg/liter) at a level similar to that of the original strain, ZJU01 (Fig. 3C). The finding supported the practicability of the strategy of using both the ΦC31- and VWB-based systems

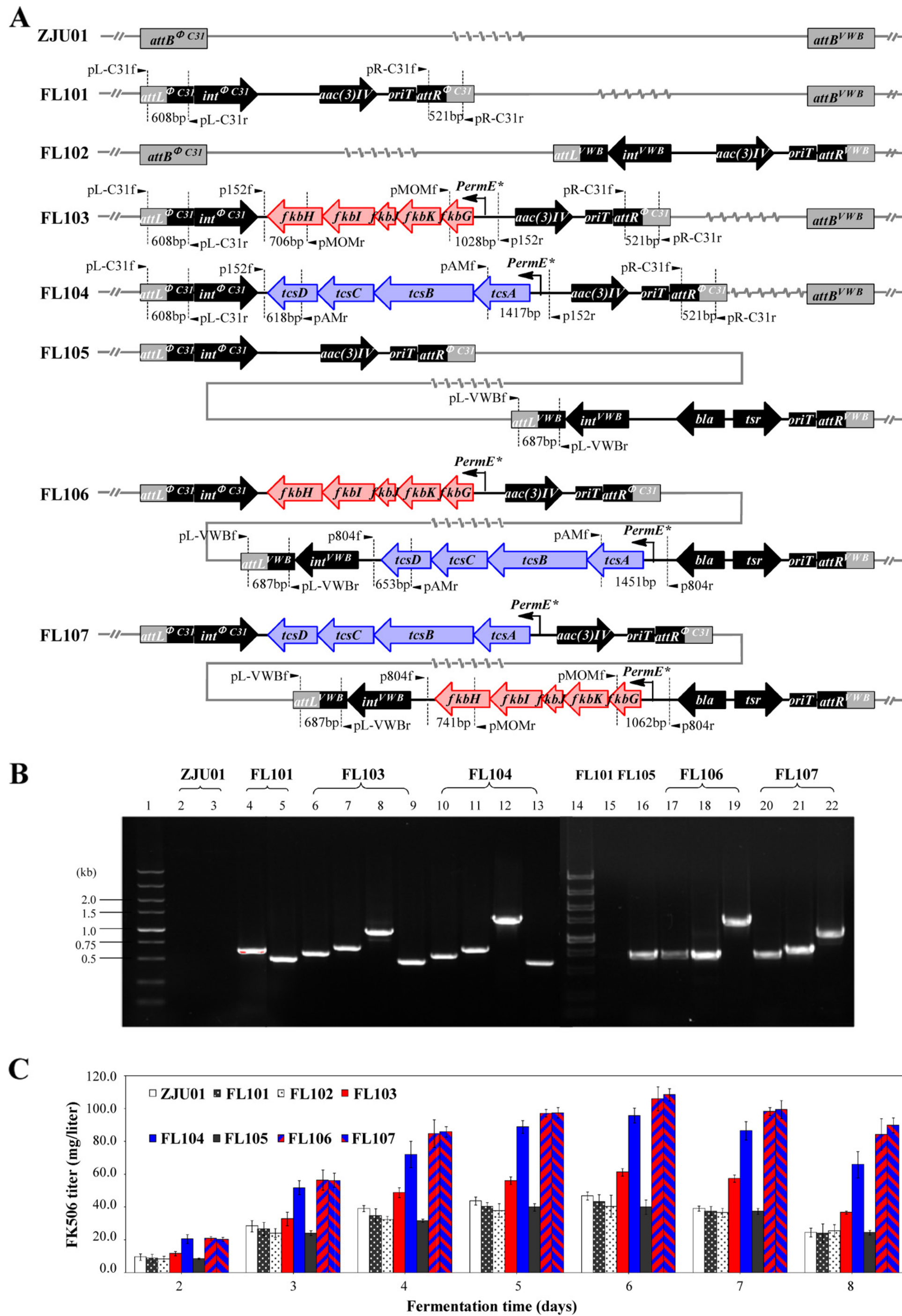

FIG 3 Duplication of the MOM-ACP and AM-CoA pathway-specific genes via two actinophage (ΦC31 and VWB) integrase-mediated recombination systems for improving FK506 production. (A) Genotypes of the FK506 producer ZJU01 and its derivatives. The primer sets are labeled with their predicted fragment sizes. (B) Validation of the genotypes by PCR amplification. The primers used to amplify individual PCR products are illustrated in Fig. 2A. (C) FK506 time course titers in the fermentations of ZJU01 and the recombinant strains. The data are the average values of at least three series of three parallel tests, and the error bars represent the standard deviations.

for recombination, given that pTA804 is compatible with pSET152 in *S. tsukubaensis*.

To increase the expression of both MOM-ACP- and AM-CoA-forming genes, we inserted the *tcsABCD* cassette into pTA804 to yield pFL106, which was introduced into FL103 by conjugation to generate the recombinant strain FL106 (containing the *fkbGHIJK* and *tcsABCD* cassettes together). The genotype of this strain was confirmed as *attL*$^{\Phi C31}$-linear pFL103-*attR*$^{\Phi C31}$-*attL*^{VWB}-linear pFL106-*attR*^{VWB} (Fig. 3A and B). After 6 days of fermentation, FK506 titers (105.9 ± 7.3 mg/liter) increased approximately 125%

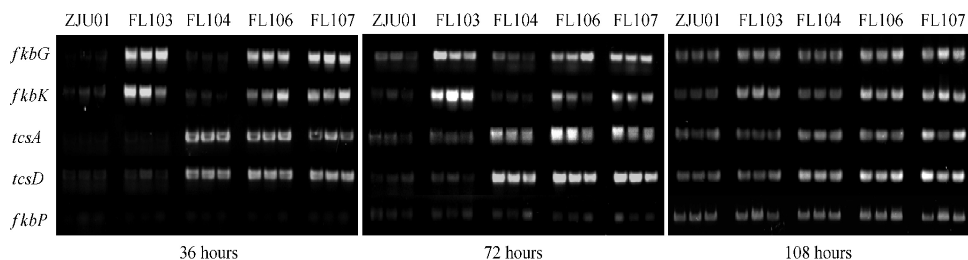

FIG 4 Semiquantitative RT-PCR analysis of the transcript levels of the *fkbGHIJK* and *tcsABCD* cassettes compared with that of *fkbP*. For each strain, the experiments were carried out on three independent isolates after 36, 72, and 108 h of fermentation. A 102-bp *fkbG* fragment and a 102-bp *fkbK* fragment were amplified to represent the *fkbGHIJK* transcript level, while a 106-bp *tcsA* fragment and a 103-bp *tcsD* fragment were amplified to represent the *tcsABCD* transcript level; a 102-bp *fkbP* fragment served as an internal control.

over that of ZJU01 (Fig. 3C). Alternatively, we constructed pFL107, a pTA804 derivative carrying the *fkbGHIJK* cassette. pFL107 was introduced into FL104 to produce a recombinant strain, FL107, whose genotype was then verified by PCR amplification and DNA sequencing as $attL^{\Phi C31}$-linear pFL104-$attR^{\Phi C31}$-$attL^{VWB}$-linear pFL107-$attR^{VWB}$ (Fig. 3A and B). FL107 showed an ability to produce FK506 similar to that of FL106 (Fig. 3C), with a maximal FK506 yield (107.9 ± 2.8 mg/liter) in fermentation on day 6. These results indicated 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*.

Transcriptional assay of the *fkbGHIJK* and *tcsABCD* cassettes by RT-PCR amplification. To correlate the overexpression of the MOM-ACP- and AM-CoA-coding genes with the duplication of the corresponding gene cassettes, we performed transcriptional analysis by semiquantitative reverse transcription (RT)-PCR amplification, with the transcription level of the NRPS gene *fkbP* as an internal control. Because the AM-CoA pathway-specific genes *tcsABCD* are organized in a contiguous form without a terminator, they can be transcribed into one polycistronic mRNA. We chose a 106-bp fragment within the first gene, *tcsA*, and a 103-bp fragment within the final gene, *tcsD*, to represent the transcription level of the AM-CoA biosynthetic genes. After reconstruction of the *fkbGHIJK* cassette, *fkbG* and *fkbHIJK* at the insertion locus were supposed to be transcribed into the same polycistronic mRNA. We chose here a 102-bp fragment within the first gene, *fkbG*, and a 102-bp fragment within the last gene, *fkbK*, to illustrate the transcription level of the MOM-ACP biosynthetic genes.

Indeed, RT-PCR analyses at the different time points in fermentation clearly indicated that the transcription levels of the genes coding for the MOM-ACP and AM-CoA biosynthetic pathways had apparently increased in FL103 and FL104, respectively (Fig. 4). This strongly supported the idea that the enhancement of pathway-specific gene transcription leads to the improvement in FK506 production. Compared to the original strain, ZJU01, the two recombinants, FL106 and FL107, with a higher yield of FK506 production, also exhibited the increased transcription levels of both the *tcsABCD* and *fkbGHIJK* cassettes in all parallel assays (Fig. 4).

Effect on FK520 production of duplication of the MOM-ACP and AM-CoA cassettes. *S. tsukubaensis* also has the ability to produce a minor product, FK520 (ascomycin), which differs from FK506 in structure only by the C-21 substitution of an ethyl group instead of an allyl group (Fig. 1A). This difference is dependent on the corresponding AT domain of the PKS system in substrate utilization, which selects ethylmalonyl (EM)-CoA as the extender unit for producing FK520 or AM-CoA for producing FK506 (20). To evaluate the relevance of FK520 with FK506 in production during the engineering process, the recombinant strains FL103, FL104, FL106, and FL107, as well as the original strain, ZJU01, were fermented and then subjected to HPLC quantification (Fig. 5). In FL103, the yield of FK520 (9.3 ± 0.6 mg/liter versus 7.4 ± 0.7 mg/liter for ZJU01) increased along with FK506, showing a ratio of FK520 to FK506 of 1:6.6 (a similar ratio of 1:6.3 was found in ZJU01). This is consistent with the prediction that the duplication of *fkbGHIJK* enhances the formation of MOM-ACP, the extender unit shared by the biosyntheses of FK520 and FK506. In FL104, the yield of FK520 (11.1 ± 0.9 mg/liter) increased; however, the growth rate was lower than that in FL103, giving a ratio of FK520 to FK506 of 1:8.6. Thus, the duplication of *tcsABCD* may mainly enhance the supply of AM-CoA, the extender unit used

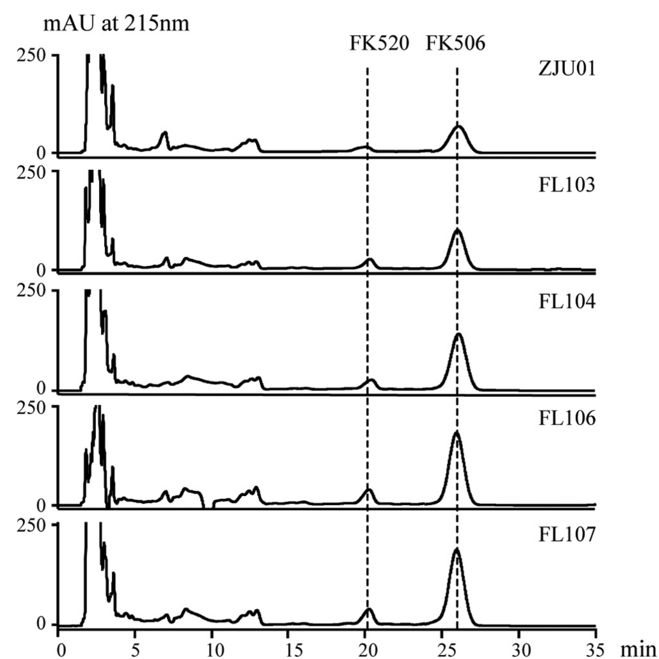

FIG 5 HPLC analysis of FK506 and FK520 from day 6 fermentation cultures of the *S. tsukubaensis* strains ZJU01, FL103, FL104, FL106, and FL107. AU, arbitrary units.

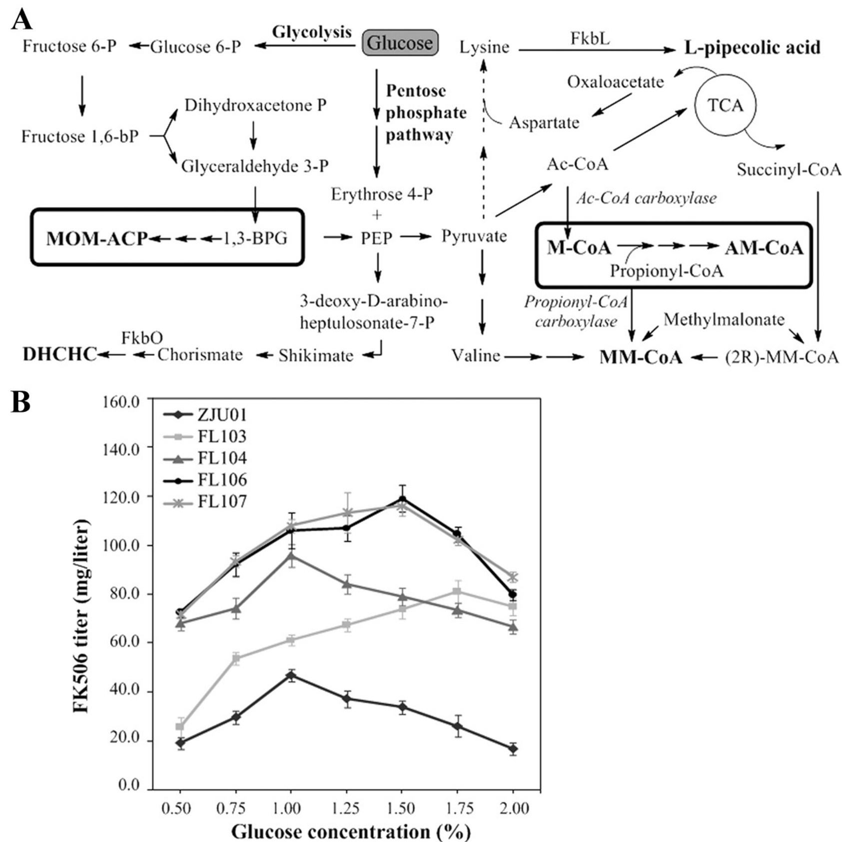

FIG 6 Glucose-dependent profile of FK506 production by the *S. tsukubaensis* strains ZJU01, FL103, FL104, FL106, and FL107. (A) Overview of glucose metabolism in relation to FK506 biosynthesis. The boxed areas represent the MOM-ACP and AM-CoA pathways. (B) FK506 titers of ZJU01 and its recombinant strains FL103, FL104, FL106, and FL107 on day 6 with respect to different glucose concentrations. The data are the average values calculated from three independent isolates of parallel tests in triplicate, and the error bars represent the standard deviations.

only in FK506 formation, providing a great potential to optimize its purity and production at the fermentation stage. In this case, the increase of FK520 production could be due to the promiscuity of the CCR protein TcsC in activity, which might accept crotonyl-CoA as the substrate to produce EM-CoA via reductive carboxylation for FK520 biosynthesis, as proposed previously (17). In line with the above, the yields of FK520 in FL106 (13.6 ± 1.0 mg/liter) and in FL107 (13.8 ± 0.7 mg/liter) also increased, showing a comprehensive effect of duplicating both *fkbGHIJK* and *tcsABCD* to give a similar ratio of FK520 to FK506 of 1:7.8.

Further improvement of FK506 production by optimizing the glucose concentration. In regard to FK506 production, the fermentation culture of *S. tsukubaensis* in this study contained soybean oil and L-lysine, which provide the precursors for FK506 biosynthesis through cellular metabolism, including M-CoA, MM-CoA, and L-pipecolic acid (7, 16, 32, 36). Glucose, one of the most commonly used carbon sources for fermentative production, also serves as a direct source to biosynthesize the precursors of various secondary metabolites. However, when the culture is grown in a high concentration of glucose (or other easily assimilated carbohydrates, such as xylose and sucrose), the production of these metabolites is usually repressed (1, 25). Although the mechanism has yet to be understood, it was proposed that this repression effect may be related to glucose transport and phosphorylation (25). An overview of glucose metabolism relevant to FK506 biosynthesis is illustrated in Fig. 6A.

Because MOM-ACP biosynthesis utilizes 1,3-BPG, which is a direct product of glycolysis (Fig. 6A), as its substrate (Fig. 1C), we reasoned that the overexpression of MOM-ACP biosynthetic enzymes would accelerate the depletion of intracellular 1,3-BPG. This depletion could consequently increase the glycolytic flux and render the cells more resistant to high concentrations of glucose. To test this hypothesis, strains were fermented in cultures with glucose concentrations that varied from 0.5% to 2.0%. FK506 production by ZJU01 and FL104 (the strain overexpressing AM-CoA biosynthetic genes alone) exhibited similar trends in response to the increased glucose concentration, showing maximal titers at 1.0% glucose and a linear decrease afterward (Fig. 6B). Production in ZJU01 decreased to about 35% of the maximum level in the culture that contained 2.0% glucose. Interestingly, for the strains overexpressing MOM-ACP biosynthetic genes, either alone or together with the AM-CoA genes, the FK506 production profiles were quite different (Fig. 6B). FK506 production by FL103 (the strain overexpressing MOM-ACP genes alone) increased almost linearly before the glucose concentration reached 1.75% (80.9 ± 4.7 mg/liter), while FL106 (119.1 ± 5.4 mg/liter) and FL107 (116.2 ± 4.1 mg/liter) (the strains overexpressing both MOM-ACP and AM-CoA genes) showed maximum FK506 production at 1.5% glucose. Consequently, FK506 production by FL106 and FL107 increased approximately 10% when the glucose concentration of the culture changed from 1.0% to 1.5%.

DISCUSSION

As a common approach for improving polyketide production, the genetic enhancement of the precursor supply has proven to be effective and promising for optimizing the strains for natural product biosynthesis. The intracellular pools of M-CoA and MM-CoA, which are the most widespread extenders and usually have low concentrations in cells (24), are frequently enriched by engineering to improve the production of desired polyketides. For example, the overexpression of acetyl (Ac)-CoA carboxylase, which diverts Ac-CoA away from the tricarboxylic acid (TCA) cycle and directs it toward M-CoA, resulted in a significant increase in actinorhodin production (27). The overexpression of the MM-CoA mutase pathway led to a 50% increase in erythromycin production by *Saccharopolyspora erythraea* (26), as well as a 50% increase in FK506 production by *S. clavuligerus* CKD1119 (19). The overexpression of propionyl-CoA carboxylase combined with the supplementation of the culture with propionate caused an 80% increase in rapamycin production by *S. hygroscopicus* ATCC 29253 (13).

M-CoA and MM-CoA metabolism is known to provide universal substrates independently of the polyketide biosynthetic pathways in microorganisms; however, FK506 biosynthesis requires two unusual pathway-specific building blocks, MOM-ACP and AM-CoA, raising the question of whether their formation in cells can be a bottleneck to production improvement. Here, the overexpression of a minimal set of genes that are responsible for the biosynthesis of either MOM-ACP or AM-CoA enhances the production of FK506 in the recombinant strains. The data suggest that the intracellular formation of the unusual PKS extender units is likely to be the rate-limiting factor and can be rationally engineered to improve FK506 production. It is noteworthy that only one molecule of AM-CoA is incorporated into the FK506 scaffold, yet the production in FL104 upon duplicating the AM-CoA biosynthetic genes increased by approximately 100% over that in ZJU01. It is likely that for the original strain, AM-CoA biosynthesis has a lower turnover number than PKS and thus is unable to provide sufficient precursors for FK506 biosynthesis.

Current successes in generating high-producing strains via site-specific recombination mainly rely on the application of the actinophage ΦC31 system (13, 19, 26, 27, 34). However, with this system, our ability to evaluate the comprehensive effect of the genetic enhancement of both the MOM-ACP and AM-CoA supplies was limited. The characterization of new site-specific recombination systems provided us with an exciting possibility that diverse genetic tools could be used at the same time for the stable expression of recombinant constructs in candidate strains. Considering that most actinomycetes are sensitive to apramycin and thiostrepton, the newly constructed vector pTA804 (VWB based) carrying ampicillin and thiostrepton resistance genes may serve as a useful tool that complements the plasmid pSET152 (ΦC31 based) for genetic manipulation in actinomycetes. The strategy of utilizing two actinophage (ΦC31 and VWB)-based recombination systems provides a convenient platform for a rapid evaluation of the gene dose effect in *S. tsukubaensis*, which facilitates the simultaneous overexpression of the MOM-ACP and AM-CoA pathways. The two systems can operate independently and exhibit a comprehensive effect, as exemplified by those in FL106 and FL107, to enhance the transcriptional levels of *fkbGHIJK* and *tcsABCD* to further improve the production of FK506.

The strategy described here could also be applicable and useful for other secondary metabolites that contain pathway-specific building blocks, as exemplified by ansatomycin, midecamycin, tautomycin, and oxazolomycin, the biosyntheses of which share a route to build the methoxy group at the β-carbon of the growing polyketide chain via the incorporation of MOM-ACP as the extender unit by PKSs (4, 14). To put it into practice, we developed a method using two distinct site-specific recombination systems in the FK506 producer *S. tsukubaensis*. These studies broaden the repertoire of actinophage-based vectors and allow the engineering of *Actinomyces* in a multiple-site-specific recombination manner.

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REFERENCES

1. **Abbanat D, Maiese W, Greenstein M.** 1999. Biosynthesis of the pyrroindomycins by *Streptomyces rugosporus* LL-42D005; characterization of nutrient requirements. J. Antibiot. **52**:117–126.
2. **Andexer JN, et al.** 2011. Biosynthesis of the immunosuppressants FK506, FK520, and rapamycin involves a previously undescribed family of enzymes acting on chorismate. Proc. Natl. Acad. Sci. U. S. A. **108**:4776–4781.
3. **Bierman M, et al.** 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene **116**:43–49.
4. **Carroll BJ, et al.** 2002. Identification of a set of genes involved in the formation of the substrate for the incorporation of the unusual "glycolate" chain extension unit in ansamitocin biosynthesis. J. Am. Chem. Soc. **124**:4176–4177.
5. **Combes P, Till R, Bee S, Smith MCM.** 2002. The streptomyces genome contains multiple pseudo-*attB* sites for the (phi)C31-encoded site-specific recombination system. J. Bacteriol. **184**:5746–5752.
6. **Craig NL.** 1988. The mechanism of conservative site-specific recombination. Annu. Rev. Genet. **22**:77–105.
7. **Gatto GJ, Boyne MT, Kelleher NL, Walsh CT.** 2006. Biosynthesis of pipecolic acid by RapL, a lysine cyclodeaminase encoded in the rapamycin gene cluster. J. Am. Chem. Soc. **128**:3838–3847.
8. **Gatto GJ, McLoughlin SM, Kelleher NL, Walsh CT.** 2005. Elucidating the substrate specificity and condensation domain activity of FkbP, the FK520 pipecolate-incorporating enzyme. Biochemistry **44**:5993–6002.
9. **Gold BG.** 2000. Neuroimmunophilin ligands: evaluation of their therapeutic potential for the treatment of neurological disorders. Expert Opin. Investig. Drugs. **9**:2331–2342.
10. **Goranovic D, et al.** 2010. Origin of the allyl group in FK506 biosynthesis. J. Biol. Chem. **285**:14292–14300.
11. **Gust B, Challis GL, Fowler K, Kieser T, Chater KF.** 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. U. S. A. **100**:1541–1546.
12. **Gust B, et al.** 2004. Lambda red-mediated genetic manipulation of antibiotic-producing *Streptomyces*. Adv. Appl. Microbiol. **54**:107–128.
13. **Jung WS, et al.** 2011. A combined approach of classical mutagenesis and rational metabolic engineering improves rapamycin biosynthesis and provides insights into methylmalonyl-CoA precursor supply pathway in *Streptomyces hygroscopicus* ATCC 29253. Appl. Microbiol. Biotechnol. **91**:1389–1397.
14. **Kato Y, et al.** 2002. Functional expression of genes involved in the biosynthesis of the novel polyketide chain extension unit, methoxymalonyl-acyl carrier protein, and engineered biosynthesis of 2-desmethyl-2-methoxy-6-deoxyerythronolide B. J. Am. Chem. Soc. **124**:5268–5269.
15. **Kieser T, Bibb MJ, Buttner MJ, Chater KF, Howood DA.** 2000. Practical Streptomyces genetics. John Innes Foundation, Norwich, United Kingdom.
16. **Kim HS, Park YI.** 2007. Lipase activity and tacrolimus production in *Streptomyces clavuligerus* CKD 1119 mutant strains. J. Microbiol. Biotechnol. **17**:1638–1644.

17. **Kosec G, et al.** 2012. Novel chemobiosynthetic approach for exclusive production of FK506. Metab. Eng. **14**:39–46.
18. **Liu J, et al.** 1991. Calcineurin is a common target of cyclophilin-cyclosporine-A and FKBP-FK506 complexes. Cell **66**:807–815.
19. **Mo S, Ban YH, Park JW, Yoo YJ, Yoon YJ.** 2009. Enhanced FK506 production in *Streptomyces clavuligerus* CKD1119 by engineering the supply of methylmalonyl-CoA precursor. J. Ind. Microbiol. Biotechnol. **36**: 1473–1482.
20. **Mo S, et al.** 2011. Biosynthesis of the allylmalonyl-CoA extender unit for the FK506 polyketide synthase proceeds through a dedicated polyketide synthase and facilitates the mutasynthesis of analogues. J. Am. Chem. Soc. **133**:976–985.
21. **Motamedi H, Cai SJ, Shafiee A, Elliston KO.** 1997. Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK506. Eur. J. Biochem. **244**: 74–80.
22. **Motamedi H, Shafiee A.** 1998. The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK506. Eur. J. Biochem. **256**:528–534.
23. **Ostash B, Makitrinskyy R, Walker S, Fedorenko V.** 2009. Identification and characterization of *Streptomyces ghanaensis* ATCC14672 integration sites for three actinophage-based plasmids. Plasmid **61**:171–175.
24. **Park JW, Jung WS, Park SR, Park BC, Yoon YJ.** 2007. Analysis of intracellular short organic acid-coenzyme A esters from actinomycetes using liquid chromatography-electrospray ionization-mass spectrometry. J. Mass Spectrom. **42**:1136–1147.
25. **Ramos I, et al.** 2004. Glucose kinase alone cannot be responsible for carbon source regulation in *Streptomyces peucetius* var. *caesius*. Res. Microbiol. **155**:267–274.
26. **Reeves AR, et al.** 2007. Engineering of the methylmalonyl-CoA metabolite node of *Saccharopolyspora erythraea* for increased erythromycin production. Metab. Eng. **9**:293–303.
27. **Ryu YG, Butler MJ, Chater KF, Lee KJ.** 2006. Engineering of primary carbohydrate metabolism for increased production of actinorhodin in *Streptomyces coelicolor*. Appl. Environ. Microbiol. **72**:7132–7139.
28. **Schwecke T, et al.** 1995. The biosynthetic gene-cluster for the polyketide immunosuppressant rapamycin. Proc. Natl. Acad. Sci. U. S. A. **92**:7839–7843.
29. **Sekurova ON, et al.** 2004. *In vivo* analysis of the regulatory genes in the nystatin biosynthetic gene cluster of *Streptomyces noursei* ATCC 11455 reveals their differential control over antibiotic biosynthesis. J. Bacteriol. **186**:1345–1354.
30. **Shen B, Hutchinson CR.** 1996. Deciphering the mechanism for the assembly of aromatic polyketides by a bacterial polyketide synthase. Proc. Natl. Acad. Sci. U. S. A. **93**:6600–6604.
31. **Sierra-Paredes G, Sierra-Marcuno G.** 2008. Ascomycin and FK506: pharmacology and therapeutic potential as anticonvulsants and neuroprotectants. CNS Neurosci. Ther. **14**:36–46.
32. **Singh BP, Behera BK.** 2009. Regulation of tacrolimus production by altering primary source of carbons and amino acids. Lett. Appl. Microbiol. **49**:254–259.
33. **Van Mellaert L, Mei L, Lammertyn E, Schacht S, Anne J.** 1998. Site-specific integration of bacteriophage VWB genome into *Streptomyces venezuelae* and construction of a VWB-based integrative vector. Microbiology**144**:3351–3358.
34. **Wu JQ, et al.** 2011. Toward improvement of erythromycin A production in an industrial *Saccharopolyspora erythraea* strain via facilitation of genetic manipulation with an artificial *attB* site for specific recombination. Appl. Environ. Microbiol. **77**:7508–7516.
35. **Wu K, Chung L, Revill WP, Katz L, Reeves CD.** 2000. The FK520 gene cluster of *Streptomyces hygroscopicus var. ascomyceticus* (ATCC 14891) contains genes for biosynthesis of unusual polyketide extender units. Gene **251**:81–90.
36. **Yoon YJ, Choi CY.** 1997. Nutrient effects on FK-506, a new immunosuppressant, production by *Streptomyces* sp. in a defined medium. J. Ferment. Bioeng. **83**:599–603.