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Targeted Gene Disruption and Functional Complementation of Cytochrome P450 Hydroyxlase Involved in Cyclosporin A Hydroxylation in *Sebekia benihana*

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A cyclic undecapeptide-family natural product, cyclosporin A (CyA), which is one of the most valuable immunosuppressive drugs, is produced nonribosomally by a multifunctional cyclosporin synthetase enzyme complex in a filamentous fungal strain named *Tolypocladium niveum.* Previously, structural modifications of cyclosporins such as a region-specific hydroxylation at the 4th *N*-methyl leucine in a rare actinomycetes called *Sebekia benihana* were reported to lead to dramatic changes in their bioactive spectra. However, the reason behind this change could not be determined since a system to genetically manipulate *S. benihana* has not yet been developed. To address this limitation, in this study, we utilized the most commonly practiced gene manipulation techniques including conjugation-based foreign gene transfer-and-expression as well as targeted gene disruption to genetically manipulate *S. benihana*. Using these optimized genetic manipulation systems, a putative cytochrome P450 hydroxylase (CYP) gene named CYP506, which is involved in CyA hydroxylation in *S. benihana*, was specifically disrupted and genetically complemented. The *S. benihana*ΔCYP506 exhibited a significantly reduced CyA hydroxylation yield as well as considerable yield restoration by functional complementation of the *S. benihana* CYP506 gene, suggesting that the genetically manipulated *S. benihana* CYP mutant strains may serve as a more efficient bioconversion host for various valuable metabolites including CyA.

Keywords: Cytochrome P450 hydroxylase, cyclosporin A, *Sebekia benihana*, targeted gene disruption

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Cytochrome P450 hydroxylase (CYP) enzymes belong to a superfamily of heme-containing monooxygenases, which catalyze remarkably diverse bioconversion reactions of several endogenous substrates such as fatty acids, steroids, and cholesterol, as well as environmental xenobiotics including carcinogens and mutagens [5, 12]. Regio- and stereo-selective CYPs are highly attractive proteins in the field of biotechnology owing to the possibility of CYP-driven bioconversion of numerous structurally diverse natural and synthetic compounds [6].

Streptomycetes and their physiologically related actinomycetes are the most important industrial microorganisms for the biosynthesis of many valuable secondary metabolites, as well as a source of various bioconversion enzymes including CYPs [4]. A rare actinomycete called *Sebekia benihana* was reported to be an important bacterium for CYP-driven natural product bioconversion, since this strain possesses unique regiospecific CYP activities toward cyclosporin, monensin, and nigericin [2, 7, 10, 13]. The actinomycetes CYP is known to transfer to the substrate one oxygen atom from a CYP-bound oxygen molecule and one hydrogen atom from NAD(P)H, which is regenerated by the ferredoxinferredoxin reductase electron transfer system [8].

A cyclic undecapeptide cyclosporin A (CyA), which is one of the most valuable immunosuppressive drugs, is produced nonribosomally by a multifunctional cyclosporin synthetase enzyme complex in the filamentous fungus *Tolypocladium niveum.* Various structural modifications of cyclosporins including a regiospecific hydroxylation at *N*-methyl leucines of CyA lead to dramatic changes in its bioactive spectra. Previously, six novel CYP genes from *S. benihana* were identified and functionally expressed using an ermE* promoter-containing *Streptomyces* expression vector in a heterologous host, *S. lividans* [9, 11]. Whereas

some *S. benihana* CYPs showed strong hydroxylation activities toward 7-ethoxycoumarin, regiospecific hydroxylation at *N*-methyl leucines of CyA was detected in the recombinant *S. lividans* containing the *S. benihana* CYP506, its adjacent ferredoxin, and the *S. coelicolor* ferredoxin reductase genes [10]. *S. benihana* CYP506 was believed to play a critical role in a regiospecific CyA hydroxylation process; however, this could not be genetically confirmed because of the lack of an available genetic manipulation system for *S. benihana*. Here, we report the establishment of a conjugation-based foreign gene transfer-and-expression system as well as a targeted gene disruption method for the genetic manipulation of *S. benihana*. Using these optimized genetic manipulation systems, the *S. benihana* CYP506 gene was shown to play a major role in a regiospecific CyA bioconversion process, not by direct hydroxylation of CyA but rather through indirect CYP interactions in *S. benihana*.

Materials and Methods

Bacterial Strains and Culture Conditions

Escherichia coli DH5α was used as a cloning host. Plasmids were propagated in *E. coli* ET12567 (*dam2*, *dcm2*, *hrdM)* to obtain unmethylated DNA for transformation into *S. benihana*. *E. coli* was grown in Luria–Bertani (LB) broth and maintained on LB agar medium at 37°C, supplemented with the appropriate antibiotics when needed. *S. benihana* (KCTC 9610) obtained from the Korean Collection for Type Cultures (KCTC, Korea) was cultured on GSMY (glucose 0.7%, yeast extract 0.45%, malt extract 0.5%, soluble starch 1.0%, and calcium carbonate 0.005%) at 28°C with constant shaking at 200 rpm for 3 days. This was followed by cell harvest and total DNA isolation. *S. benihana* exconjugants were supplemented with apramycin (25 μg/ml) or hygromycin (25 μg/ml).

Construction of Recombinant Integrative Plasmid pCYP506

For functional overexpression of the CYP506 gene in *S. benihana*, a 1.2-kb DNA fragment including the entire CYP506 gene and its adjacent ferredoxin gene was amplified by PCR, using the pESK506-E1plasmid as a template with the primer pair described previously [11], with the replacement of restriction sites between *Bam*HI–*Hin*dIII with *Bam*HI–*Xba*I restriction sites. PCR was performed in a final volume of 20 μl containing 0.4 M of each primer, 0.25 mM of each of the 4 dNTPs, 1 μl of extracted DNA, 1 U of *Ex Taq* polymerase (TaKaRa, Japan) in its recommended reaction buffer, and 10% DMSO. Amplifications were performed in a Thermal Cycler (BioRad, USA) according to the following profile: 30 cycles of 60 s at 95°C, 60 s at 55°C, and 70 s at 75°C. The amplified PCR product was analyzed by electrophoresis in 1% (w/v) agarose gel and purified using a DNA extraction kit (COSMO, Korea). The PCR products were ligated into a pGEM T-easy vector (Takara, Japan), followed by complete nucleotide sequencing confirmation by Genotech Korea. The PCR products were finally subcloned into the pSET152 derivative integrative plasmid containing a strong constitutive promoter, ermE*, and the resulting plasmid was designated pCYP506. The pCYP506 plasmid was introduced into *S. benihana via* the conjugation method, followed by apramycin (*apr*) selection.

E. coli–S. benihana Interspecies Conjugation

A three-day-old 10-ml *S. benihana* culture in RARE3 medium (glucose 10 g, yeast extract 4 g, malt extract 10 g, Bacto-peptone 2 g, $MgCl_2 \cdot H_2O$ 2 g, glycerol 5 g in 1 l of distilled water) was diluted with 90 ml of fresh broth and grown for an additional 24 h at 28°C. The culture was then centrifuged, resuspended in 10 ml of fresh medium, homogenized, and fragmented by sonication (2 cycles of 3 s on and 4 s off using an ultrasonic generator, Ulsso Hi-tech, Korea). After further growth for 20 h at 28°C, the culture was centrifuged again, resuspended in fresh RARE3 medium, and sonicated as before. The donor *E. coli* strain ET12567/pUZ8002, carrying the disruption construct (pMJ922), was prepared according to standard *E. coli–Streptomyces* conjugation [3]. During each mating experiment, donor and recipient cells were mixed and plated on modified ISP4 medium. After 20 h of incubation at 28°C, each plate was overlaid with apramycin and nalidixic acid. The *S. benihana* exconjugant colonies usually appeared after 1–2 weeks of incubation.

Construction of CYP506 Gene-Disrupted *S. benihana* Mutant

The *S. benihana* mutant strain was constructed using the PCR-targeted gene-disruption system according to the general method detailed by Gust *et al*. [3]. An apramycin resistance gene/*oriT* cassette for the replacement of the CYP506 gene was amplified using pIJ773 as a template and the following primers: forward primer (5'-atgacggagcccccg tacaccgtgaccgcgctgcccaccaATTCCGGG GATCCGTCGACC-3') and reverse primer (5'-ctactcctt gtcccacgcg accgggagccggtgcacgccgTGTAGGCTGGAGCTGCTTC-3'). The lower-case type represents 40 nt homologous extensions to the DNA regions inside the CYP506 gene. The resultant PCR product was used to target the cosmid 9–22 containing the CYP506 gene in *E. coli* BW25113/pIJ790. The mutated cosmid was transferred to *S. benihana* by conjugation *via* ET12567/pUZ8002, and the desired mutants, which were the products of double crossovers, were identified by screening for colonies that were apramycin resistant but kanamycin sensitive. The double-crossover exconjugants were selected using the standard apr^R/kan^S method [3], followed by confirmation of both *S. benihana* and *S. benihana*ΔCYP506 genomic DNAs by both PCR and Southern-blot hybridization. Three different PCR primers used to confirm the double-crossover recombinants were CYP506 test primer F (5'-acgaatccagcagcatcaccctg-3'), CYP506 test primer R (5'-acgcaacatagcttttgctggga-3'), and *oriT* test primer F (5'-gaattcagcgtgacatcattctgtgg-3'), which is in the apr^R/*oriT* cassette.

Complementation of CYP506 Gene-Disrupted *S. benihana* Mutant

The recombinant integrative plasmid pCYP506 was used for complementation of the CYP506-disrupted mutant (*S. benihana*ΔCYP506). Since the *S. benihana*ΔCYP506 strain was already apramycin resistant owing to CYP506 gene disruption, the apramycin resistance gene was changed to the hygromycin resistance gene from the pMS82 plasmid using a PCR-targeted gene disruption system and named pMMBL301. Finally, hygromycin was used for selection of the recombinant *S. benihana* ΔCYP506/pMMBL301.

HPLC Analysis for CyA Hydroxylation in *S. benihana*

The metabolites were analyzed using HPLC equipped with a photodiode array detector and symmetry C18 reverse-phase column, with a two-buffer gradient system; 25% methanol (buffer A) and 100% acetonitrile (buffer B). One cycle of the buffer B gradient was programmed to start at 40% for 4 min, 61% for 20 min, 100% for 40 min, and 40% for 45 min. The column temperature was maintained at 60°C. Cyclosporine and its derivatives were monitored at 210 nm. The flow rate was maintained at 1.0 ml/min and the injection volume was 20 µl.

RESULTS AND DISCUSSION

E. coli–*S. benihana* Interspecies Conjugation and CYP506 Expression

Although the *E. coli*-driven interspecies conjugation method has been successfully used in most *Streptomyces* species, some rare actinomycetes strains including *S. benihana* are extremely recalcitrant to the typical interspecies conjugation approach. As a first step of *S. benihana* genetic manipulation, the most commonly practiced *E. coli*-driven interspecies conjugation method was modified and optimized in *S. benihana* to facilitate gene transfer and targeted gene disruption. Among the many steps conducted during the *E. coli*–*S. benihana* interspecies conjugation procedure, the harvesting period and sonication-driven fragmentation of the recipient *S. benihana* mycelia seemed to be the most critical factor in maximizing the conjugation efficiency.

Since regiospecific CyA hydroxylation in *S. lividans* was previously observed when the *S. benihana* CYP506 gene was coexpressed with its adjacent ferredoxin gene and the *S. coelicolor* ferredoxin reductase gene, *S. benihana* CYP506 may play a critical role in the process of CyA hydroxylation [10]. To further verify its biological significance *via* CYP506 functional overexpression in *S. benihana*, the entire coding region of the CYP506 gene including its 3' downstream ferredoxin gene locus was amplified by PCR and inserted into the *Streptomyces* integrative vector pSET152, which contained a strong constitutive promoter, ermE* (named pCYP506, Fig. 1A), followed by conjugation to *S. benihana*. The *S. benihana* exconjugant strain containing the CYP506 overexpression construct (*S. benihana*/pCYP506) was genetically confirmed by PCR analysis (Fig. 1B).

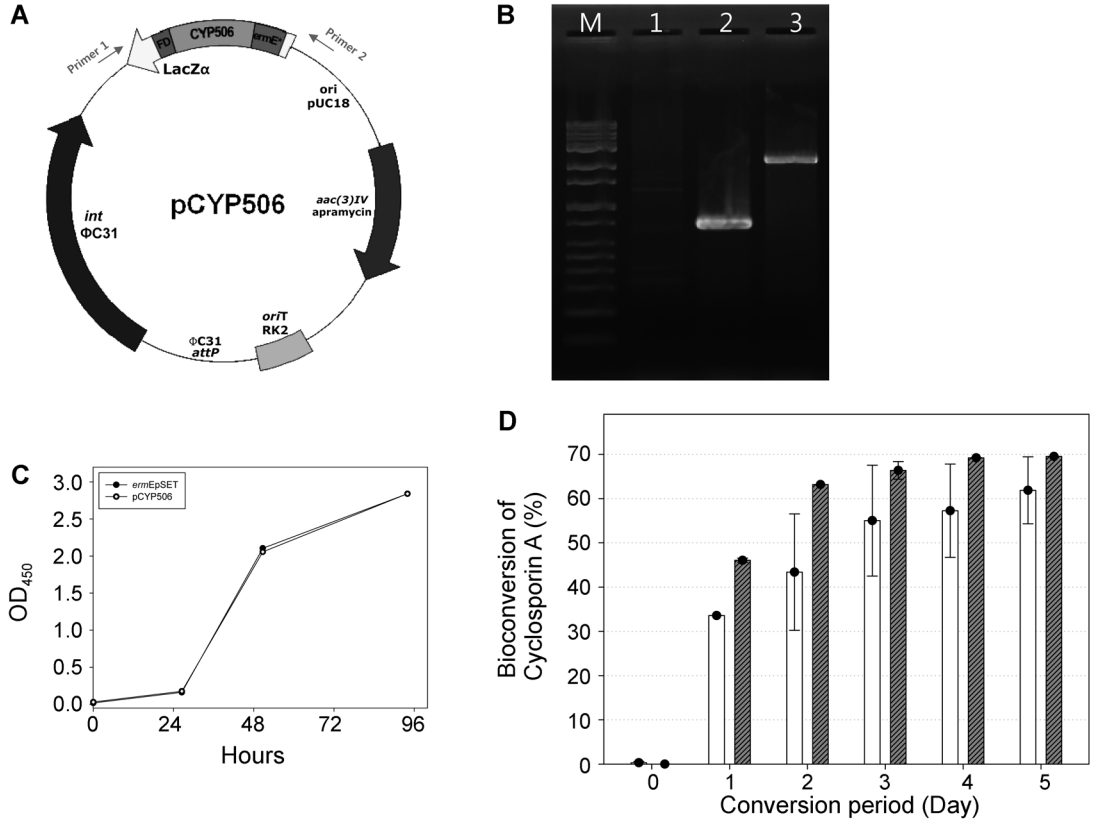

Fig. 1. Plasmid construction and gene CYP506 expression in *S. benihana* and its CYP506-overexpressing exoconjugant strain.
A. Map of the CYP506 and ferredoxin genes in the *Streptomyces* expression vector, *ermE**pSET152 (pCYP506). **B**. PCR with the DNA samples of five-day-old GSMY liquid cultures. Lane M, 1 kb size marker; lane 1, genomic DNA of wild-type strain; lane 2, *ermE**pSET152 plasmid; lane 3, genomic DNA of *S. benihana*/pCYP506. **C**. Growth of *S. benihana* and *S. benihana*/pCYP506 measured by optical density at 450 nm. **D**. Conversion yield of CyA hydroxylation measured by quantitative HPLC analysis. The vertical bars show the standard deviation values of the mean of three separate determinations.

The selected *S. benihana*/pCYP506 and the control (*S. benihana* containing only the vector) strains were first cultured in GSMY medium for 3 days and both showed comparable growth patterns (Fig. 1C). Both strains were then further cultured for 5 more days after the addition of 100 mg/l of CyA as the hydroxylation substrate into the culture. HPLC analyses of samples isolated during the 5-day conversion culture period revealed that a slightly higher CyA hydroxylation yield on average was observed in the *S. benihana*/pCYP506 compared with the *S. benihana* strain containing the vector alone (Fig. 1D). This result implies that the conversion stimulation effect of the additional CYP506 gene expression in *S. benihana* may not be very significant owing to the possible saturation of CYP506 gene expression and/or limitation of the CYP50-specific electron transfer partners [1].

Disruption of CYP506 Gene in *S. benihana*

To further verify the biological significance of CYP506-driven CyA hydroxylation, inactivation of the CYP506 gene was performed by slightly modifying the *Streptomyces* PCR-targeted gene disruption system [2]. Cosmid 9–22 was replaced with an apramycin resistance/*oriT* cassette, generating pMJ922, which was introduced into *S. benihana* by conjugative gene transfer (Fig. 2A). Construction of the CYP506-deleted mutant (named *S. benihana*ΔCYP506) was confirmed by both PCR and Southern hybridization analysis. The expected 1.3 kb PCR-amplified band was observed in genomic DNA samples isolated from *S. benihana*, and a band at the expected size (1.63 kb) was observed in genomic DNA samples isolated from *S. benihana*ΔCYP506 (Fig. 2B). A PCR-amplified fragment with the expected size (0.47 kb), amplified using an alternative PCR primer pair designed to detect the apramycin resistance gene/*oriT* cassette, was observed only in the *S. benihana*ΔCYP506 strain (Fig. 2B). Moreover, two different theoretically calculated *Sal*I digestion patterns were observed in Southern hybridization with *S. benihana* and *S. benihana*ΔCYP506 (Fig. 2C), indicating that not only was the CYP506 gene in *S. benihana* specifically disrupted

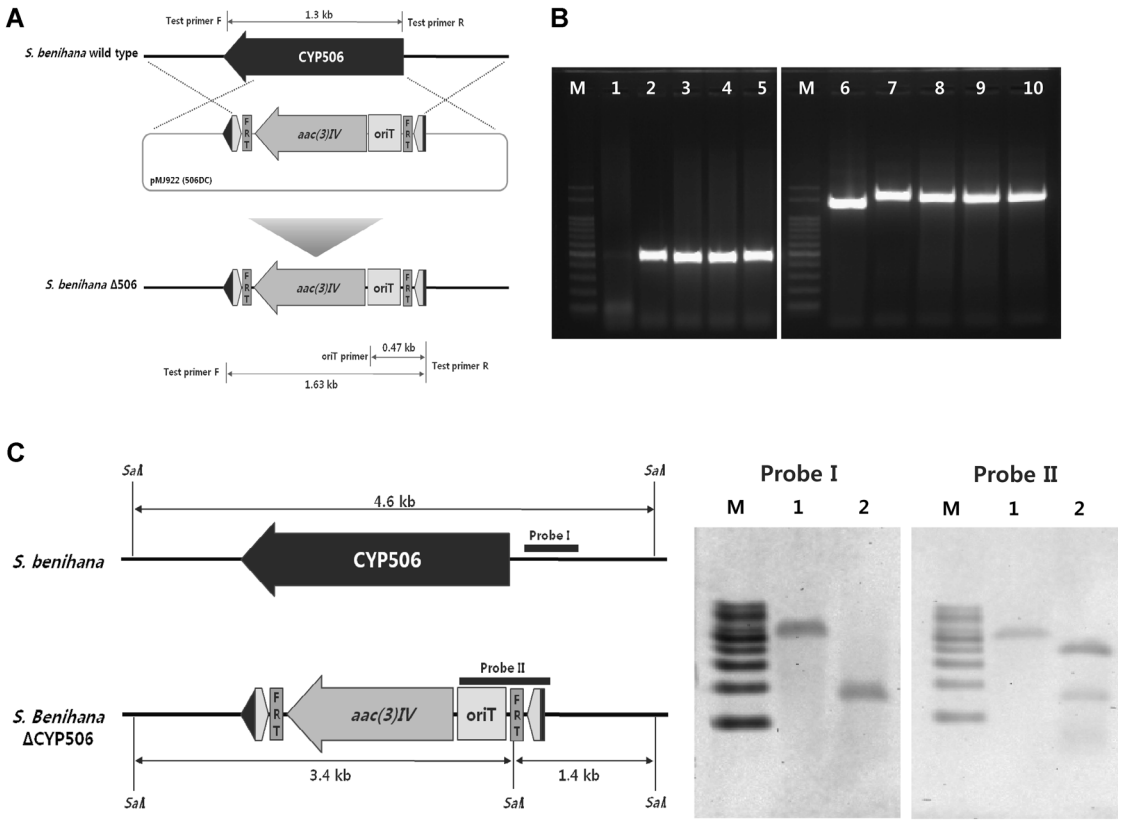

Fig. 2. Gene replacement of the CYP506 gene.

A. Schematic representation of PCR-targeted gene replacement disruption of CYP506 and apramycin resistance *(apr^R)*/*oriT*. **B**. Confirmation of the constructed *S. benihana*ΔCYP506 mutant by PCR analysis. Lanes: M, 1 kb ladder; 1and 6, *S. benihana* wild type genomic DNA; 2 and 7, CYP506 gene disruption construct (pMJ922); 3–5 and 8–10, *S. benihana*ΔCYP506 genomic DNA. In lanes 1–5, PCR was performed with test F and R primers; in 6–10, PCR was performed with oriT and test R primers. **C**. Confirmation of the constructed *S. benihana*ΔCYP506 mutant by Southern hybridization. A diagrammatic representation of the digest and probe binding sites is shown to the left of the blot; Lanes: 1, *S. benihana* wild-type genomic DNA; 2, *S. benihana*ΔCYP506 genomic DNA.

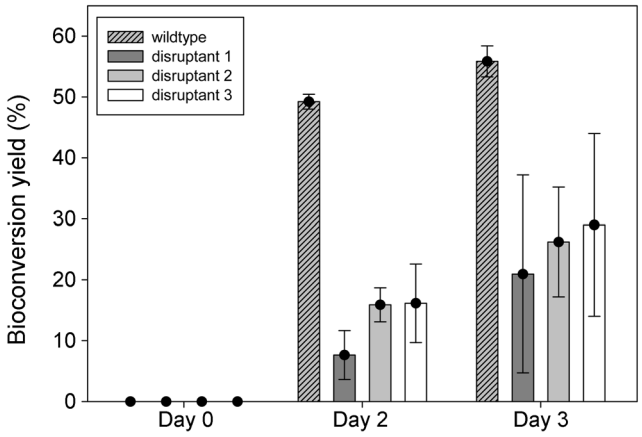

Fig. 3. Conversion yield of CyA hydroxylation in *S. benihana* and three differently isolated *S. benihana*ΔCYP506 strains.
The vertical bars show the standard deviation values of the mean of three separate determinations.

as expected but also that the *Streptomyces* PCR-targeted gene disruption system was efficiently applicable to a rare actinomycetes including *S. benihana*.

S. benihana wild-type and three independently isolated *S. benihana*ΔCYP506 mutant strains were cultured and optimized for CyA hydroxylation, followed by HPLC quantification. Although all three *S. benihana*ΔCYP506 mutant strains exhibited significantly reduced CyA hydroxylation yields (about 50–70% reduction) compared with the wild type under the same culture condition, CyA hydroxylation was not completely eradicated (Fig. 3).

Complementation of CYP506 Gene in *S. benihana*

In order to further prove that the inactivation of the CYP506 gene was indeed responsible for CyA hydroxylation, genetic complementation of *S. benihana*ΔCYP506 was performed by expressing CYP506 under the constitutive control of the *ermE** promoter, pCYP506. Since the *S. benihana*ΔCYP506 strain was already apramycin resistant as a result of CYP506 gene disruption, the apramycin resistance gene was changed to a hygromycin resistance gene from the pMS82 plasmid using a PCR-targeted gene disruption system (named pMMBL301) (Fig. 4A). Hygromycin was used for the selection of recombinant strains bearing a complementation plasmid. The plasmid was conjugated to *S. benihana*ΔCYP506, and the resulting *S. benihana*ΔCYP506/pMMBL301 strain was confirmed by PCR analysis (Fig. 4B). As expected, HPLC analysis confirmed that the CYP506-driven CyA hydroxylation activity was restored to considerable levels in the *S. benihana*ΔCYP506 strain carrying pMMBL301 (Fig. 4C). These results suggest that the CYP506 is one of the major CYPs involved in a regiospecific CyA bioconversion process, even though it might not be the only major CyA hydroxylation enzyme in *S. benihana*. In summary, CYP506 could be one of several

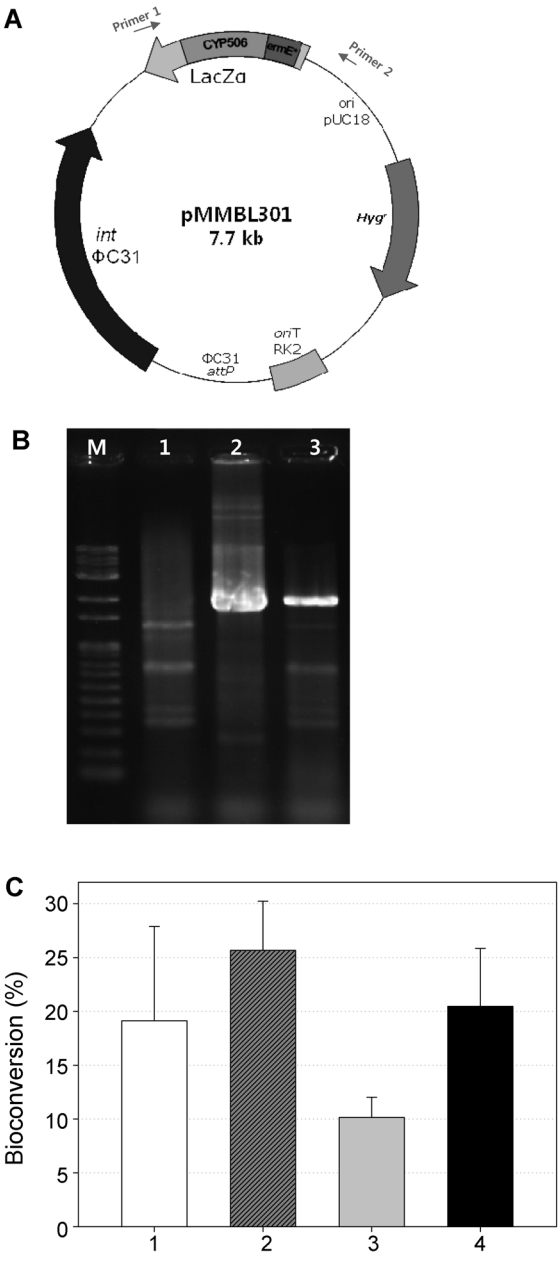

Fig. 4. Genetic complementation of *S. benihana*ΔCYP506 to prove the role of CYP506 in CyA hydroxylation.
A. Construction of the recombinant plasmid pMMBL301 for CYP506 complementation in the *S. benihana*ΔCYP506 strain. **B**. PCR analysis using the genomic DNA samples of *S. benihana*ΔCYP506 and *S. benihana*ΔCYP506/pMMBL301 strains. Lane M, 1 kb size marker; lane 1, *S. benihana*ΔCYP506; lane 2, pMMBL301; lane 3, *S. benihana*ΔCYP506/pMMBL301. **C**. Comparison of CyA hydroxylation yield (%) in *S. benihana* wild type (1), *S. benihana* /pCYP506 (2), *S. benihana*ΔCYP506 (3), and *S. benihana*ΔCYP506/pMMBL301 (4). The vertical bars show the standard deviation values of the mean of three separate determinations.

CYPs involved in CyA hydroxylation and might play a major role in a regiospecific CyA bioconversion process, not by direct hydroxylation of CyA but rather through indirect CYP interactions in *S. benihana*.

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