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A novel regio-specific cyclosporin hydroxylase gene revealed through the genome mining of *Pseudonocardia autotrophica*

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Abstract The regio-specific hydroxylation at the 4th *N*-methyl leucine of the immunosuppressive agent cyclosporin A (CsA) was previously proposed to be mediated by a unique cytochrome P450 hydroxylase (CYP), CYP-sb21 from the rare actinomycetes *Sebekia benihana.* Interestingly, a different rare actinomycetes species, *Pseudonocardia autotrophica*, was found to possess a different regio-selectivity, the preferential hydroxylation at the 9th *N*-methyl leucine of CsA. Through an in silico analysis of the whole genome of *P. autotrophica*, we describe here the classification of 31 total CYPs in *P. autotrophica*. Three putative CsA CYP genes, showing the highest sequence homologies with CYP-sb21, were successfully inactivated using PCR-targeted gene disruption. Only one knock-out mutant, ΔCYP-pa1, failed to convert CsA to its hydroxylated forms. The hydroxylation activity of CsA by CYP-pa1 was confirmed by CYP-pa1 gene complementation as well as heterologous expression in the CsA non-hydroxylating *Streptomyces coelicolor*. Moreover, the cyclosporine regio-selectivity of CYP-pa1 expressed in the ΔCYP-sb21 *S. benihana* mutant strain was also confirmed unchanged through cross complementation. These results show that preferential regio-specific hydroxylation at the 9th *N*-methyl leucine of CsA is carried out by a specific P450 hydroxylase gene in *P. autotrophica*, CYP-pa1, setting the stage for the biotechnological application of CsA regio-selective hydroxylation.

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Introduction

Actinomycetes are a group of soil bacteria well known for their ability to produce pharmacologically important secondary metabolites as well as various industrial enzymes including cytochrome P450 hydroxylase (CYP) [1, 15]. Bacterial CYPs belong to the superfamily of heme-containing monooxygenases that catalyze numerous types of reactions including biotransformation of natural products such as steroids, fatty acids, polyketides, and xenobiotic compounds. The catalytic abilities of CYPs are highly attractive due to their superior regio- and stereo-selectivities, thereby providing the possibility of various biotechnological applications such as hydroxylation of structurally diverse natural as well as synthetic compounds through CYP-driven bioconversion processes [5, 7, 18, 24].

A rare actinomycetes, *S. benihana*, transfers a hydroxyl group to various natural products including cyclosporine A (CsA), vitamin D3, monensin, and nigericin [3, 16, 23]. The regio-specific hydroxylation of an immunosuppressive CsA at the 4th *N*-methyl leucine is mediated by a unique CYP in *S. benihana*. Interestingly, a hydroxylated CsA derivative at the 4th *N*-methyl leucine position was reported to maintain the hair growth-promoting side effect while causing the complete loss of CsA immunosuppressive activity, implying that the regio-specific hydroxylation of a natural product could dramatically alter its biological activity [13]. Through genome sequencing and in silico analysis of *S. benihana*, the complete CYPome of *S. benihana* including 21 CYPs along with their electron transfer partners [seven ferredoxins (FDs) and four ferredoxin reductases (FDRs)],

were identified from *S. benihana*. Using *E. coli* conjugation-based CYPome-targeted disruption in *S. benihana*, each of the identified CYP, FD, and FDR genes in *S. benihana* were individually inactivated. Out of the 32 *S. benihana* CYPome mutants tested, only a single *S. benihana* CYP mutant, ΔCYP-sb21, failed to exhibit CsA hydroxylation activity, revealing that CYP-sb21 is the only essential component of the in vivo CsA regio-specific hydroxylation process in *S. benihana* [11, 12].

A different rare actinomycetes species, *P. autotrophica*, was also found to possess CsA hydroxylation activity but with the different regio-selectivity. *P. autotrophica* was reported to preferentially transfer a hydroxyl group at the 9th *N*-methyl leucine of CsA unlike the 4th *N*-methyl leucine in *S. benihana* [14]. Although the hydroxylated CsA derivative at the 9th *N*-methyl leucine position was also reported to maintain the hair growth-promoting effect, its relative biological activities comparing with the 4th position hydroxylated CsA need to be further determined. Here, we report the first identification and characterization of the CYP-sb21 orthologue in *P. autotrophica*, which shows a different CsA regio-selectivity. Through in silico analysis of the whole genome of *P. autotrophica* as well as targeted gene disruption, complementation, and heterologous expression techniques, a unique *P. autotrophica* CYP gene, CYP-pa1 (GenBank accession number KJ433554), was identified and confirmed as the gene responsible for the CsA-specific hydroxylation at the 9th *N*-methyl leucine.

Materials and methods

Bacterial strains and cultivation conditions

A rare actinomycete, *P. autotrophica* KCTC9441, was purchased from the Korea Collection for Type Cultures (KCTC, Daejeon, Korea), and cultured on GSMY medium (glucose 0.7 %, yeast extract 0.45 %, malt extract 0.5 %, soluble starch 0.75 %, and calcium carbonate 0.005 %) at 30 °C for confirmation of CsA hydroxylation. *Streptomyces coelicolor* M145 was cultured on Tryptic Soy Broth medium for confirmation of CsA hydroxylation and further cultured on YEME medium (yeast extract 0.3 %, peptone 0.5 %, malt extract 0.3 %, glucose 1 %, sucrose 34 % and $MgCl_2 \cdot 6H_2O$ 5 mM, and glycine 0.5 %, all separately autoclaved) for protoplast transformation at 30 °C. Recombinant plasmids were introduced into *E. coli* strains by transformation and electroporation. *E. coli* DH5α was used for DNA cloning, *E. coli* BL21 (DE3) was used for protein expression, ET12567 was used to avoid DNA methylation in the plasmid, and *E. coli* ET12567 harboring pUZ8002 was used for *E. coli-Streptomyces* conjugation [10].

Bioconversion assay and HPLC analysis of CsA-specific hydroxylation

The selected *P. autotrophica* strains were cultured in GSMY medium at 30 °C for 72 h, treated with CsA (50 mg/l) as the hydroxylation substrate, and further cultured for an additional 48 h. The crude compound was extracted from culture solution using ethyl acetate, the ethyl acetate evaporated, and the crude compound resuspended in methanol. The samples were analyzed using HPLC equipped with a photodiode array detector and Vydac 218TP C_{18} Column (250 × 4.6 mm 5 μm, GRACE, USA) in a two-buffer gradient system consisting of 25 % methanol (buffer A) and 100 % acetonitrile (buffer B). The column temperature was 60 °C. One cycle of buffer B gradient was programmed as follows: 40 % for 4 min, 61 % for 15 min, and 40 % for 10 min. CsA and its derivatives were monitored at 210 nm. The flow rate was 1.0 ml/min, and the injection volume was 20 μl.

Identification of the *P. autotrophica* CYPome through whole-genome mining

The *P. autotrophica* whole genome, with a total size of approximately 10 Mbp and containing more than 10,000 ORFs, was analyzed. Functions of the predicted protein-encoding genes were annotated by comparisons with the UniRef, NCBI-NR, COG, and KEGG database [2, 9, 19, 21]. Amino acid sequence alignment was conducted using Clustal W2 Multiple Sequence Alignment (EMBL-EBI).

PCR-targeted gene disruption and complementation

The *P. autotrophica* mutant strains were made using a PCR-targeted gene-disruption system as previously described [6]. An apramycin resistance gene/*oriT* cassette for the replacement of CYP genes was amplified using pIJ773 as a template and the disruption primers (Table 1S). For the functional overexpression and complementation of the CYP-pa1, CYP-pa2, and CYP-pa3 genes in *P. autotrophica*, a DNA fragment, including the entire CYP-pa gene with *Bam*HI (GGATCC) and *Xba*I (TCTAGA) sites, was obtained using CYP-pa1, CYP-pa2, or CYP-pa3 complementation primers (Table 1S). PCR was performed in a final volume of 20 μl containing 0.4 M of each primer, 0.25 mM of each of the four dNTPs, 1 μl of extracted DNA, 1 U of Ex *Taq* polymerase (Takara, Shiga, Japan) in its recommended reaction buffer, and 10 % DMSO. Amplifications were performed in a Thermal Cycler (Bio-Rad,

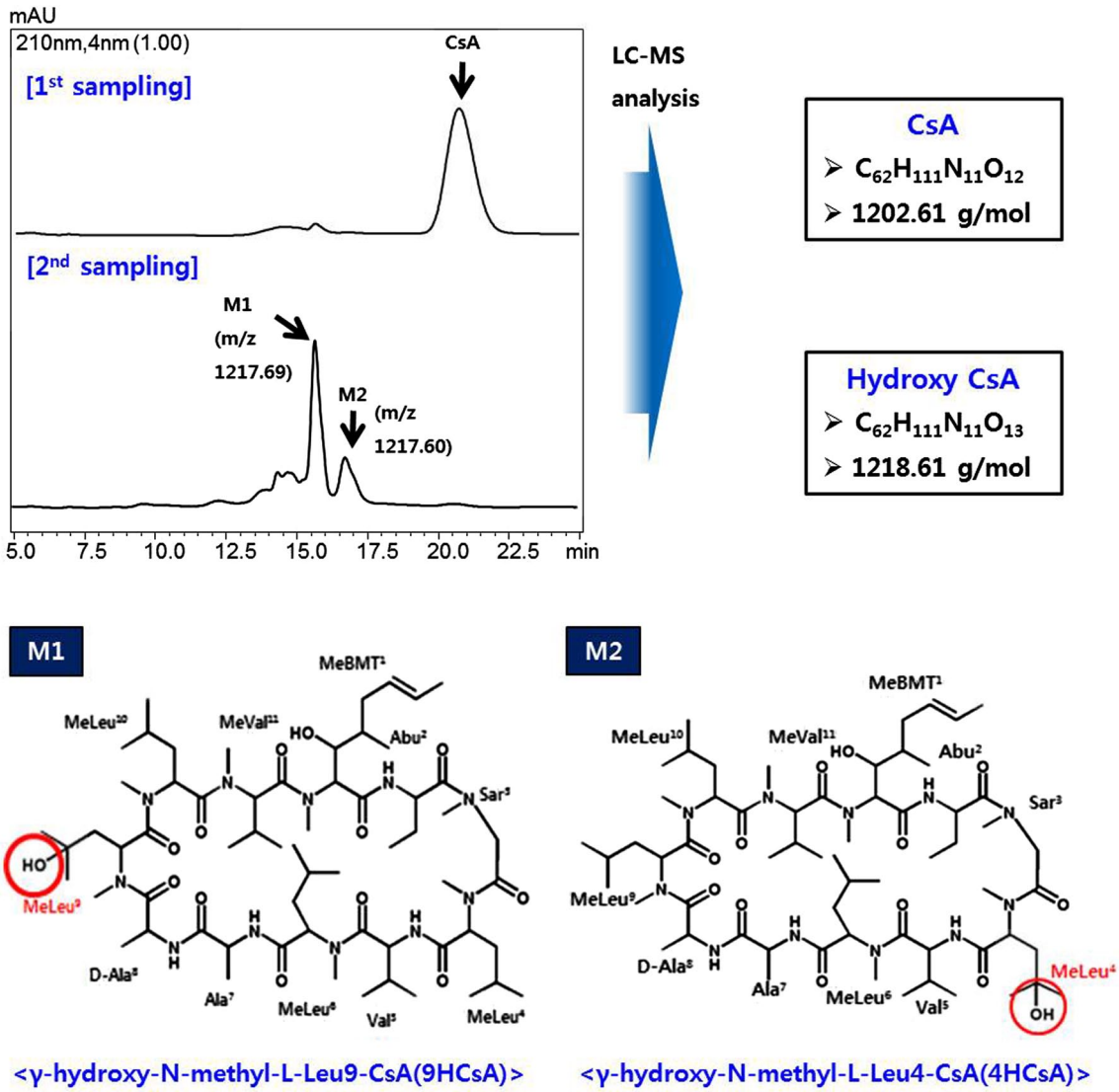

Fig. 1 Structure and HPLC profile of CsA and hydroxylated CsA in *P. autotrophica*. Their structures were confirmed by LC–MS analyses (Fig. S1)

Hercules, CA, USA) according to the following profile: 30 cycles of 60 s at 95 °C, 60 s at 58 °C, and 60 s at 75 °C. The amplified PCR product was analyzed by 1 % (w/v) agarose gel electrophoresis and purified using a DNA extraction kit (Cosmo genetech, Seoul, Korea). The PCR products were then ligated into a RBC T&A cloning vector (Real Biotech Corporation, Taipei, Taiwan). The ligated vector was completely sequenced to ensure its integrity (Macrogen, Seoul, Korea). The PCR products were finally subcloned into the pSET152-derived integration plasmid containing a strong constitutive promoter, ermE*, and hygromycin resistance gene. The resulting plasmids were designated as pCYP-pa1, pCYP-pa2, and pCYP-pa3, respectively. The pCYP-pa1 was introduced into the *P. autotrophica* wild type and CYP-pa1 disruptant via conjugation, followed by *att*B site integration and hygromycin selection.

Construction of the pHE-pa1 heterologous expression plasmid

For heterologous expression of the CYP-pa1 gene in *S. coelicolor*, a DNA fragment containing the entire CYP-pa1 gene was amplified using genomic DNA from *P. autotrophica* as a template and CYP-pa1 HE primers containing *Nde*I (CATATG) and *Eco*RI (GAATTC) restriction enzyme sites (Table 1S). The PCR products were finally subcloned into the pHSEV-1 plasmid containing an inducible *tip*A promoter [20, 22] and the resulting plasmid was designated

as pHE-pa1. The pHE-pa1 plasmid was introduced into *S. coelicolor* via the protoplast transformation method, followed by kanamycin (*kan*) selection.

Heterologous expression of the CYP-pa1 gene in *S. coelicolor*

The recombinant plasmid pHE-pa1 was used for protoplast transformation [10]. For preparation of protoplast transformation, lysozyme (1 mg/ml) was used to treat *S. coelicolor* M145 and ΔSCO5426 mutants cultured in YEME medium. The transformation was performed with pHE-pa1 and 25 % PEG 1000 solution in P buffer (sucrose 10.3 %, K_2SO_4 0.025 %, $MgCl_2{\cdot}6H_2O$ 0.202 %, trace element solution 0.2 %). The transformed *S. coelicolor* M145 and ΔSCO5426 mutant were cultured in a R2YE agar plate [sucrose 10.3 %, K_2SO_4 0.025 %, $MgCl_2{\cdot}6H_2O$ 1.012 %, glucose 1 %, casamino acids 0.01 %, agar 1.1 %, KH_2PO_4 (0.5 %) 0.1 %, $CaCl_2{\cdot}2H_2O$ (3.68 %) 0.8 %, L-proline (20 %) 0.15 %, TES buffer (5.73 %, adjusted to pH 7.2) 1 %, trace element solution 0.02 %, NaOH (1 N) 0.05 % prepared separately] and incubated at 30 °C, and Kanamycin (kan) was used for the selection of recombinants.

Results and discussion

Bioinformatic analysis of CYP-pa1 in P. autotrophica

It has been reported that the biological activities of the cyclic undecapeptide immunosuppressive CsA could be significantly altered through regio-selective hydroxylations. Previously, two rare actinomycetes strains, *S. benihana* and *P. autotrophica*, were identified as strains showing the highest CsA regio-specific hydroxylation activities at the 4th *N*-methyl leucine and the 9th *N*-methyl leucine, respectively [13]. Recently, we identified *S. benihana* CYP-sb21 as the gene responsible for the 4th position-specific CsA hydroxylation process in *S. benihana* [12]. As shown in Fig. 1, *P. autotrophica* was confirmed to preferentially transfer the hydroxyl group at the 9th over the 4th *N*-methyl leucine position of CsA. To isolate a CYP-sb21 orthologue with different CsA regio-selectivity such as 9th *N*-methyl leucine-specific CsA hydroxylation, the whole-genome sequences of *P. autotrophica* were analyzed. This revealed a total of 31 distinct CYP genes (Fig. 3SA). Among *P. autotrophica* CYPs, only three CYP genes, CYP-pa1, CYP-pa2, and CYP-pa3 were selected as putative CYP-sb21 orthologues due to these genes having the

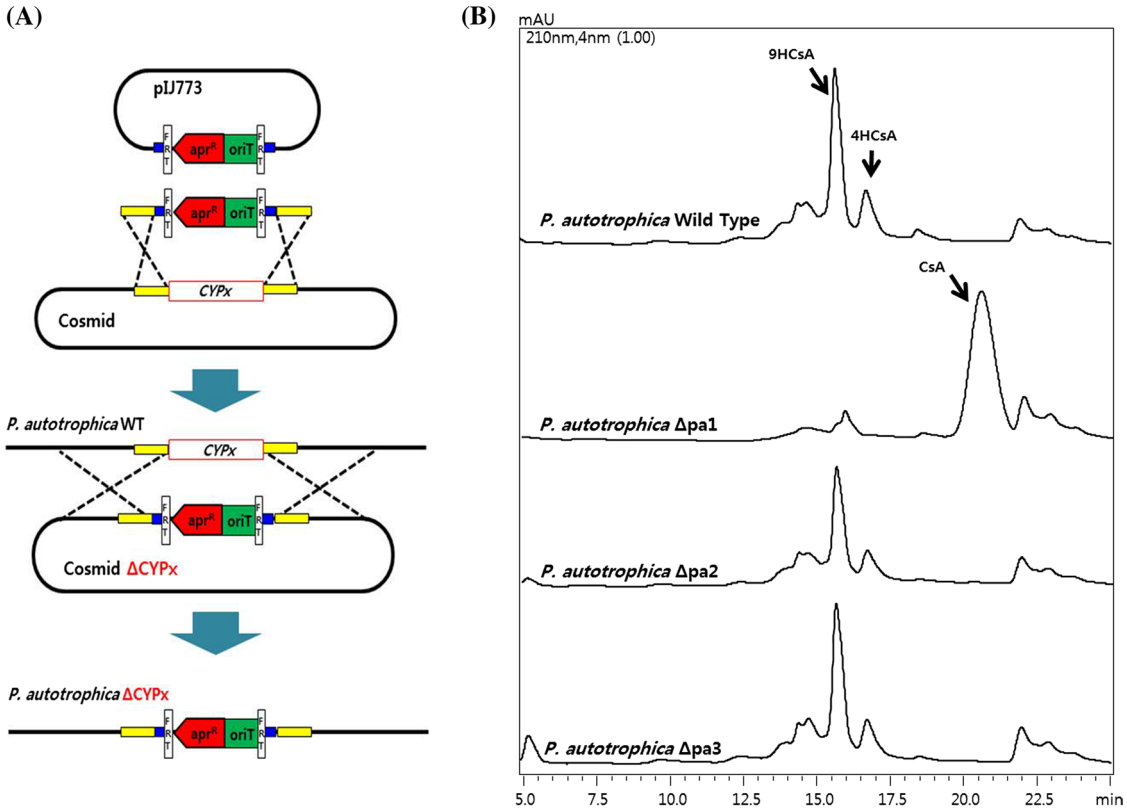

Fig. 2 **a** Schematic representation of PCR-targeted gene replacement disruption of CYPs (CYP-pa1, CYP-pa2, and CYP-pa3) with the apramycin (apr^R)/*oriT*. **b** HPLC profiles of CYP knock-out mutants

highest encoded amino acid sequence homologies with *S. benihana* CYP-sb21 (Fig. 3SA). The CYP-specific motifs, including the I-helix (oxygen-binding motif, [17]), K-helix (ferredoxin-binding motif, [8]), and cysteine heme–iron ligand (heme-binding motif, [4]) were also conserved in the *P. autotrophica* CYP genes (Fig. 3SB). CYP-pa1 showed the highest sequence homology with the CYP-sb21 (55 % amino acid identity), while CYP-pa2 and CYP-pa3 showed 47 and 46 % amino acid identities, respectively, with CYP-sb21.

Targeted CYP gene disruption in *P. autotrophica*

To identify the true CYP-sb21 orthologue responsible for the 9th *N*-methyl leucine-specific CsA hydroxylation process in *P. autotrophica*, the CYP-pa1, CYP-pa2, and CYP-pa3 genes were inactivated using a PCR-targeted gene disruption system. An apramycin resistance (*apr*^{r})/*oriT* cassette was added to the cosmids containing the target gene, which was then introduced into *P. autotrophica* by conjugative gene transfer (Fig. 2a). The construction of CYP mutants (named *P. autotrophica* ΔCYP-pa#) generated by PCR-targeted gene disruption was confirmed by PCR using CYP-pa check primers. The expected PCR-amplified bands were observed in genomic DNA samples isolated from *P. autotrophica* as well as from *P. autotrophica* ΔCYP-pa (Fig. 2S), implying that each CYP gene was specifically disrupted as expected. *P. autotrophica* and the independent *P. autotrophica* ΔCYP mutants grown in fermentation broths under optimal conditions for CsA hydroxylation were extracted using ethyl acetate, followed by HPLC quantification. As shown in Fig. 2b, only the gene disruption of CYP-pa1 completely abolished CsA regio-specific hydroxylation in *P. autotrophica*, providing strong evidence that CYP-pa1 encodes the key enzyme involved in CsA regio-specific hydroxylation.

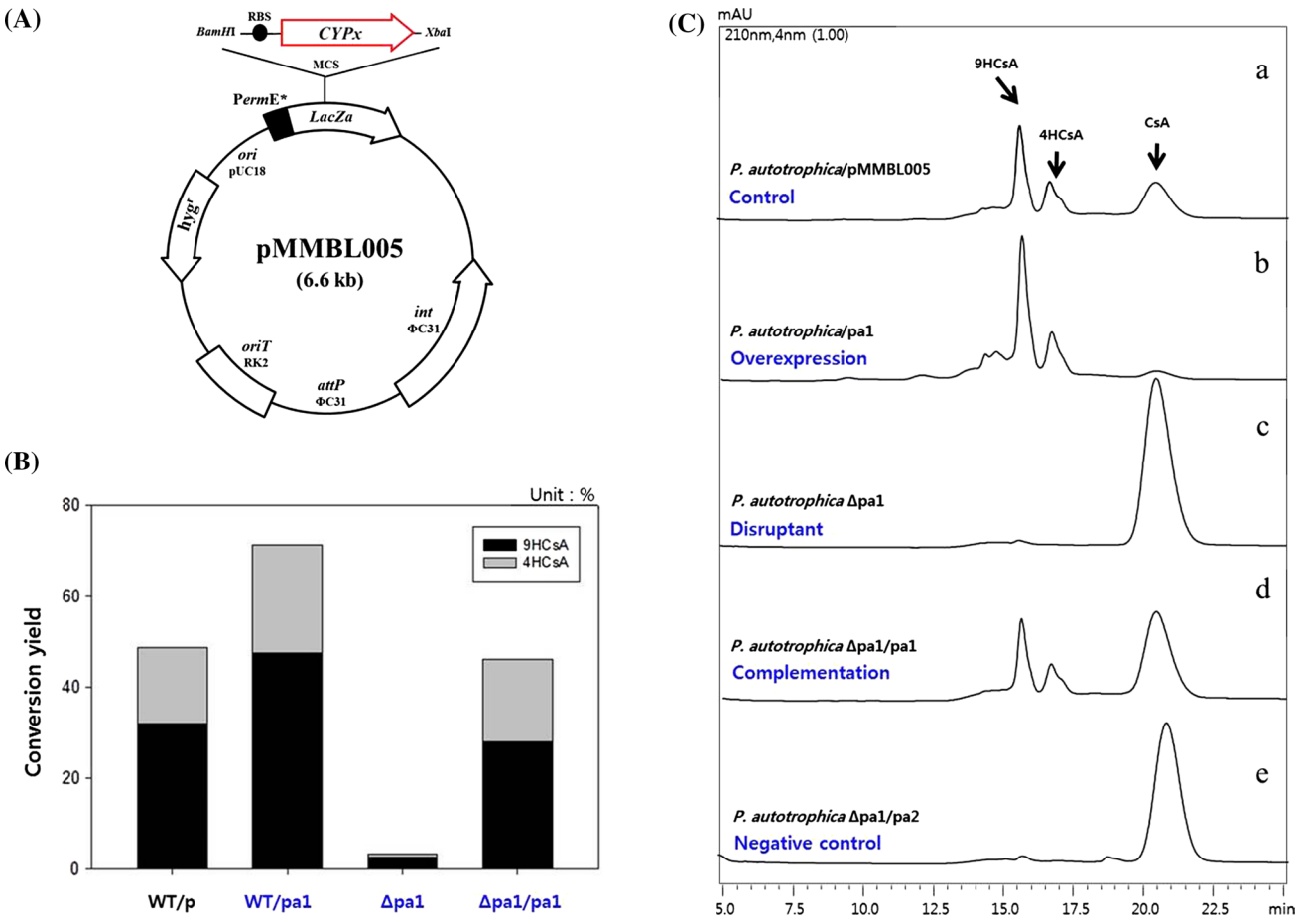

Fig. 3 Genetic overexpression and complementation of CYP-pa1 in *P. autotrophica* strains. **a** Construction of the recombinant plasmids pCYP-pa1 and pCYP-pa2 using the pMMBL005 plasmid, **b** conversion yields, and **c** HPLC profiles of CsA bioconversion in *P. autotrophica*/pMMBL005, *P. autotrophica*/pCYP-pa1, *P. autotrophica*ΔCYP-pa1, *P. autotrophica* ΔCYP-pa1/pCYP-pa1, and *P. autotrophica* ΔCYP-pa1/pCYP-pa2 strains

Complementation and overexpression of the CYP-pa1 gene in *P. autotrophica*

To further prove that the CYP-pa1 gene was responsible for CsA hydroxylation, genetic complementation of *P. autotrophica* ΔCYP-pa1 was performed. The coding sequence of the CYP-pa1 gene was cloned into a *Streptomyces* expression vector harboring the constitutive promoter *ermEp** and the hygromycin-resistant gene containing pMMBL005 (pCYP-pa1, Fig. 3a). Hygromycin was then used for the selection of recombinant strains bearing the complementation plasmid. Plasmids were independently conjugated to *P. autotrophica* wild type as well as to the ΔCYP-pa1 mutant strains. The *P. autotrophica*/pCYP-pa1 (overexpression strain) and *P. autotrophica* ΔCYP-pa1/pCYP-pa1 (complementation strain) were confirmed by PCR analyses. HPLC analysis indicated that CsA hydroxylation activity driven by CYP-pa1 in the *P. autotrophica* ΔCYP-pa1 was restored to a significant level in the *P. autotrophica* ΔCYP-pa1/pCYP-pa1 strain, while almost no CsA hydroxylation was observed in *P. autotrophica* ΔCYP-pa1 containing either pCYP-pa2 or pCYP-pa3 (Fig. 3b). Moreover, the wild-type *P. autotrophica* containing an extra copy of the CYP-pa1 gene via chromosomal integration led to an approximately 1.4-fold increase in CsA hydroxylation yield (Fig. 3c), which implies that CYP-pa1 is indeed the CYP-sb21 orthologue responsible for the 9th *N*-methyl leucine-specific hydroxylation of CsA in *P. autotrophica.*

Functional expression of the CYP-pa1 gene in a heterologous host

For the functional expression of the CYP-pa1 gene in a heterologous *Streptomyces* host, *S. coelicolor* M145 was used as an expression host based on its easy genetic manipulation characteristics as well as its previous record as an excellent host for foreign gene expression. Most importantly, the *S. coelicolor* host strain does not carry out either CsA hydroxylation or any other CsA modification. The

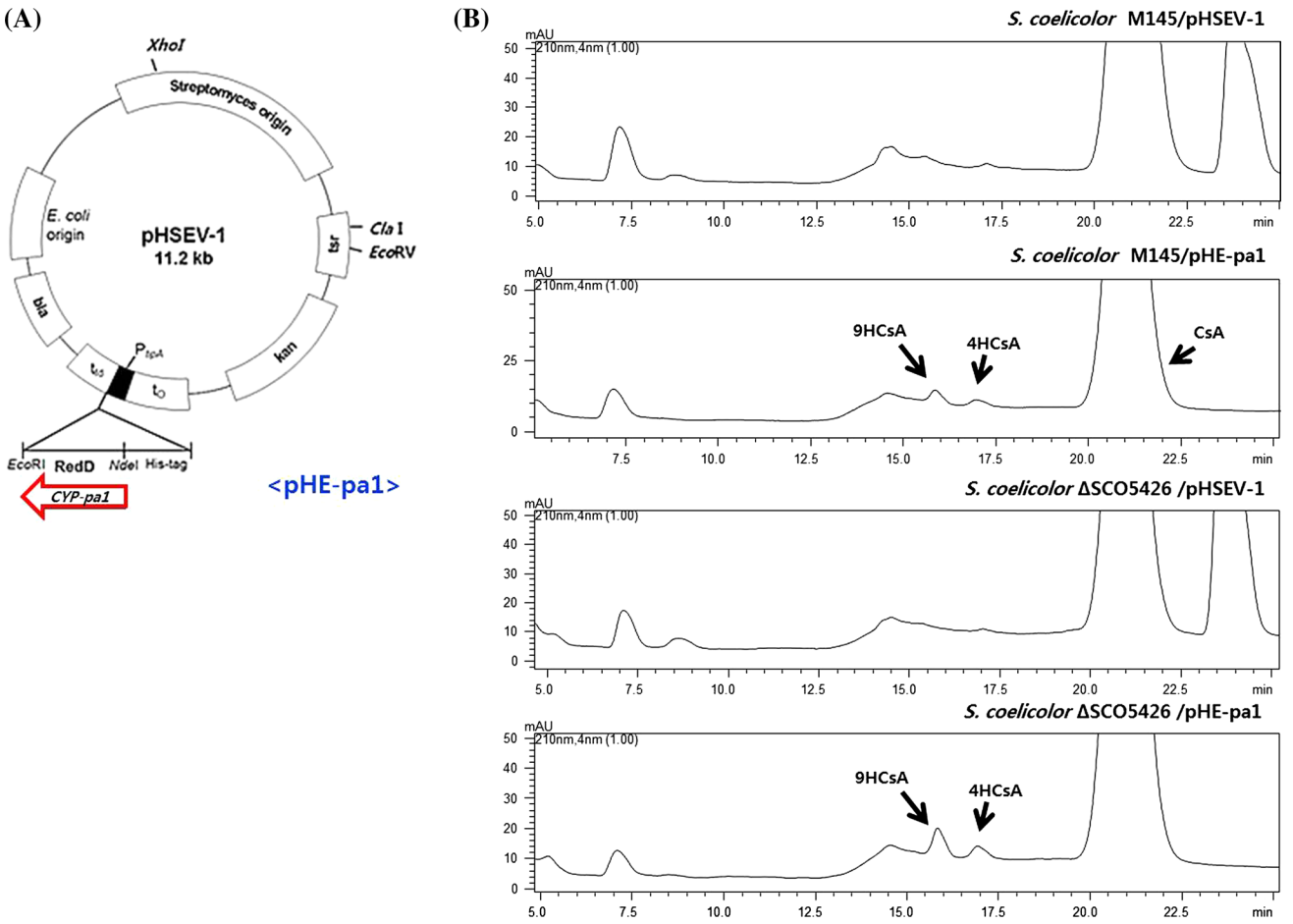

Fig. 4 **a** Map of the pHE-pa1 plasmid, a pHSEV-1 derivative. **b** The HPLC profiles of CsA metabolites with heterologous expression of CYP-pa1 in *S. coelicolor* M145/pHE-pa1 and *S. coelicolor* ΔSCO5426/pHE-pa1

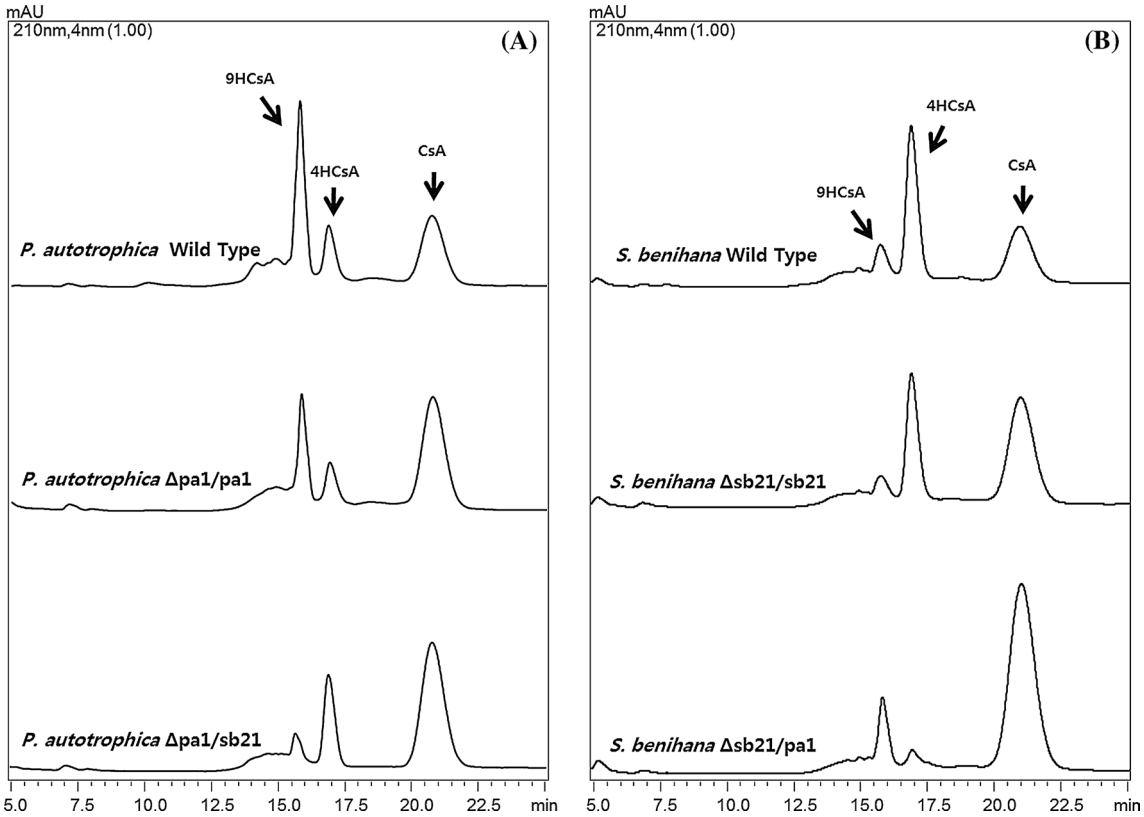

Fig. 5 HPLC profiles of cross-complementation strains **a** pCYP-sb21 in *P. autotrophica* ΔCYP-pa1 mutant and **b** pCYP-pa1 in *S. benihana* ΔCYP-sb21 mutant

S. coelicolor M145ΔSCO5426 mutant strain was specifically used for a heterologous host because of its enhanced NADPH supply [12]. The coding sequence of CYP-pa1 was cloned into the *Streptomyces* expression vector called pHSEV1 (named pHE-pa1), which harbors the inducible tipAp promoter. This pHE-pa1 construct was transformed into *S. coelicolor* M145 or M145ΔSCO5426 through PEG-assisted protoplast transformation, followed by kanamycin selection. The resulting transformant was then confirmed to be the correct recombinant strain by PCR analysis. As shown in Fig. 4, CsA hydroxylation was detected in both strains but at a much reduced conversion yield. Since some CYP-driven hydroxylations require specific reducing partners such as ferredoxin and ferredoxin reductase, appropriate reducing partners might be required for optimal *P. autotrophica* CYP-pa1-driven CsA hydroxylation.

Comparison of regio-specific hydroxylations between CYP-pa1 and CYP-sb21

As stated above, the CYP-pa1 identified from *P. autotrophica* could be the CYP-sb21 orthologue hydroxylase with different regio-selectivity. To further confirm that the CsA regio-selectivity was specifically due to the target CYP and no other factors in the host cell, cross gene complementation was performed. CYP-sb21 was expressed in the *P. autotrophica* ΔCYP-pa1 mutant strain and CYP-pa1 was expressed in the *S. benihana* ΔCYP-sb21 mutant strain. The *S. benihana* ΔCYP-sb21 strain containing pCYP-pa1 mostly exhibited 9th *N*-methyl leucine-specific hydroxylation activity, while the *P. autotrophica* ΔCYP-pa1 strain containing the pCYP-sb21 mostly showed 4th *N*-methyl leucine-specific hydroxylation activity (Fig. 5). These cross-complementation results clearly demonstrated that the CYP itself determines the hydroxylation position of the CsA with a unique regio-specificity. Although a sequence comparison between CYP-sb21 and CYP-pa1 does not currently pinpoint the specific domain or motif responsible for CsA regio-selectivity, further genetic and biochemical studies will eventually lead to a better understanding of how a large natural product like CsA could be preferentially recognized and specifically catalyzed by CYP enzymes from actinomycetes.

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