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Domain Characterization of Cyclosporin Regio-Specific Hydroxylases in Rare Actinomycetes S

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Cytochrome P450 hydroxylase (CYP) in actinomycetes plays an important role in the biosynthesis and bioconversion of various secondary metabolites. Two unique CYPs named CYP-sb21 and CYP-pa1, which were identified from *Sebekia benihana* and *Pseudonocardia autotrophica*, respectively, were proven to transfer a hydroxyl group at the 4th or 9th *N*-methyl leucine position of immunosuppressive agent cyclosporin A (CsA). Interestingly, these two homologous CYPs showed different CsA regio-selectivities. CYP-sb21 exhibited preferential hydroxylation activity at the 4th position over the 9th position, whereas CYP-pa1 showed the opposite preference. To narrow down the CYP domain critical for CsA regio-selectivity, each CYP was divided into four domains, and each domain was swapped with its counterpart from the other CYP. A total of 18 hybrid CYPs were then individually tested for CsA regio-selectivity. Although most of the hybrid CYPs failed to exhibit a significant change in regio-selectivity in the context of CsA hydroxylation, hybrid CYP-pa1 swapped with the second domain of CYP-sb21 showed a higher preference for the 9th position. Moreover, hybrid CYP-sb21 containing seven amino acids from the 2nd domain of CYP-pa1 showed higher preference for the 4th position. These results imply that the 2nd domain of CsA-specific CYP plays a critical role in CsA regio-selectivity, thereby setting the stage for biotechnological application of CsA regio-selective hydroxylation.

Keywords: Cytochrome P450, cyclosporin A, regio-selectivity, actinomycetes

Introduction

Bacterial cytochrome P450 hydroxylase (CYP) belongs to the superfamily of heme-containing monooxygenases, which catalyze the biotransformation of natural products such as steroids, fatty acids, polyketides, and xenobiotic compounds [6, 7, 12, 13]. Bacterial CYP is known to transfer a single CYP-bound oxygen atom along with a single hydrogen atom from NAD(P)H, which is regenerated by the ferredoxin (FD)-ferredoxin reductase (FDR) electron transfer system, to its substrate [4, 8, 11]. The catalytic abilities of CYPs have gained attention owing to their superior regio- and stereo-selectivities and thus hold potential for the hydroxylation of natural and synthetic compounds with diverse structures *via* CYP-driven bioconversion processes.

Several CYPs in various actinomycetes have been shown to produce pharmacologically useful secondary metabolites [2, 14]. CYPs detected in rare actinomycetes such as *Sebekia benihana* are known to introduce hydroxyl groups to secondary metabolites at certain locations [5, 13]. Among them is cyclosporin A (CsA) produced from *Tolypocladium inflatum*, which receives a hydroxyl group at its 4th amino acid, methyl leucine (γ-hydroxy-*N*-methyl-L-Leu4-CsA, 4HCsA). Although *S. benihana* CYP also introduces a hydroxyl group at the 9th methyl leucine of CsA (γ-hydroxy-*N*-methyl-L-Leu9-CsA, 9HCsA), it demonstrates a preference for the 4th amino acid position. Another actinomycete named *Pseudonocardia autotrophica* also introduces a hydroxyl group at the 4th or 9th methyl leucine, but it shows preference for the 9th methyl leucine position (LG Household and Health Care. 2007. Korean Patent

1008652110000, LG Household and Health Care. 2008. Korean Patent 1006816700000). Unlike immunosuppressant CsA, hydroxylated CsA at either the 4th or 9th position shows significantly reduced immunosuppressant function, although it retains the hair growth-stimulation side effect of CsA.

We previously identified two CsA-specific CYPs: *S. benihana* CYP-sb21 preferentially introduces a hydroxyl group at the 4th methyl leucine of CsA, whereas *P. autotrophica* CYP-pa1 prefers the 9th methyl leucine of CsA [1, 9, 10]. To identify which domain determines regio-selectivity between these two CYP genes, we divided the two CYP genes into four domains, followed by hybrid CYP gene construction by domain swapping with each other. Each hybrid CYP was then introduced into a deletion mutant of CYP-sb21 (*S. benihana* ΔCYP-sb21) or CYP-pal (*P. autotrophica* ΔCYP-pa1) in order to determine the domain responsible for CsA regio-selectivity.

Materials and Methods

Bacterial Strains and Cultivation Conditions

S. benihana KCTC9610 and *P. autotrophica* KCTC9441 were purchased from the Korea Collection for Type Cultures (KCTC, Korea). *S. benihana* wild-type and mutant strains were grown on GSMY medium [10], and *P. autotrophica* wild-type and mutant strains were grown on ISP2 medium (Difco, BD), at 30°C. *E. coli* strains were grown in Luria broth or agar at 37°C. All bacterial strains were cultured with the appropriate antibiotics. *E. coli* ET12567/pUZ8002 was used as the donor strain for *E. coli-Streptomyces* conjugation [3].

Amino Acid Sequence Alignment and 3D Structure Modeling

The amino acid sequences of CYP-sb21 and CYP-pa1 were aligned using ClustalW2 from EMBL-EBI. For protein modeling, a *P. autotrophica* vitamin D3 hydroxylase CYP template was searched against CYP-sb21 and CYP-pa1 using the Automated Model from SWISS-MODEL. Three-dimensional structure models of CYP-sb21 and CYP-pa1 were built using the template.

Construction of Mutant Genes by In-Fusion Cloning Method

All hybrid genes were constructed using an In-Fusion HD Cloning kit (Clontech, CA, USA). A linearized vector was generated using restriction enzymes (*Bam*HI, *Xba*I), and DNA fragments were generated by PCR. The linearized vector and DNA fragments were fused by In-Fusion enzyme. PCR was performed according to previously established conditions [1]. All PCR products were purified by electrophoresis on 1% agarose gel using a DNA extraction kit (Cosmo Genetech, Seoul, Korea). Primer and template DNAs used to make mutant CYP genes are shown in Table S1. Primer sequences are shown in Table S2.

Bioconversion Assay and HPLC for CsA and Its Derivatives

All actinomycete strains were cultured in 30 ml of GSMY broth at 30°C for 72 h. Then, CsA (50 mg/l) was added to the culture medium as a substrate. After an additional 48 h, 8 ml of culture was sampled. Each 8 ml of culture was extracted with one volume of ethyl acetate, and the combined organic layer was dried by rotary evaporation. The resulting residue was dissolved in 0.7 ml of methanol. The samples were analyzed by HPLC according to previously established conditions [1].

Results

Amino Acid Sequence Alignment and Domain Swapping Between CsA-Specific CYPs

We performed amino acid comparison between the two CsA-specific CYPs in order to select locations for division of the CYP-sb21 and CYP-pa1 domains (Fig. 1A). Amino acid comparison detected 55% similarity, and chosen sequences showed significant differences or amino acid insertion compared with the control group. Using SWISS-MODEL, other CYPs with known 3D structures were compared with these two CYPs in order to select a template CYP, which was identified as vitamin D3 hydroxylase from *P. autotrophica*. In comparing the expected 3D structures of the two CYPs with that of vitamin D3 hydroxylase (Fig. 1B), the CYP-sb21 and CYP-pa1 genes were subdivided into four domains. The CYP-sb21 gene was divided into 1–135, 136–225, 226–301, and 302–410, whereas the CYP-pa1 gene was divided into 1–134, 135–228, 229–304, and 305–412 (Fig. 1A).

To determine whether or not a specific domain affects regio-selectivity during hydroxylation of CsA, we altered the four domains of the two CYP genes. Using the In-fusion cloning method (Fig. 1C), a total of 14 hybrid CYP genes were generated in the actinomycete chromosome integration pMMBL005 vector, and the nomenclature was as follows: CYP-SP001 ~ CYP-SP007 and CYP-PS001 ~ CYP-PS007 (Fig. 1D). Each hybrid CYP sequence was analyzed to confirm the domain changes. To determine whether or not CsA is hydroxylated by hybrid CYPs, each hybrid CYP gene was introduced into *S. benihana* ΔCYP-sb21 or *P. autotrophica* ΔCYP-pa1 by the *E. coli*-actinomycetes interspecies conjugation method.

CsA Hydroxylation by Hybrid CYPs

To determine whether or not regio-selectivity of hydroxylation differs between wild-type and hybrid CYPs, recombinant strains along with *S. benihana* and *P. autotrophica* wild-type strains were tested for CsA bioconversion. Since bioconversion rates for all recombinant *S. benihana* ΔCYP-

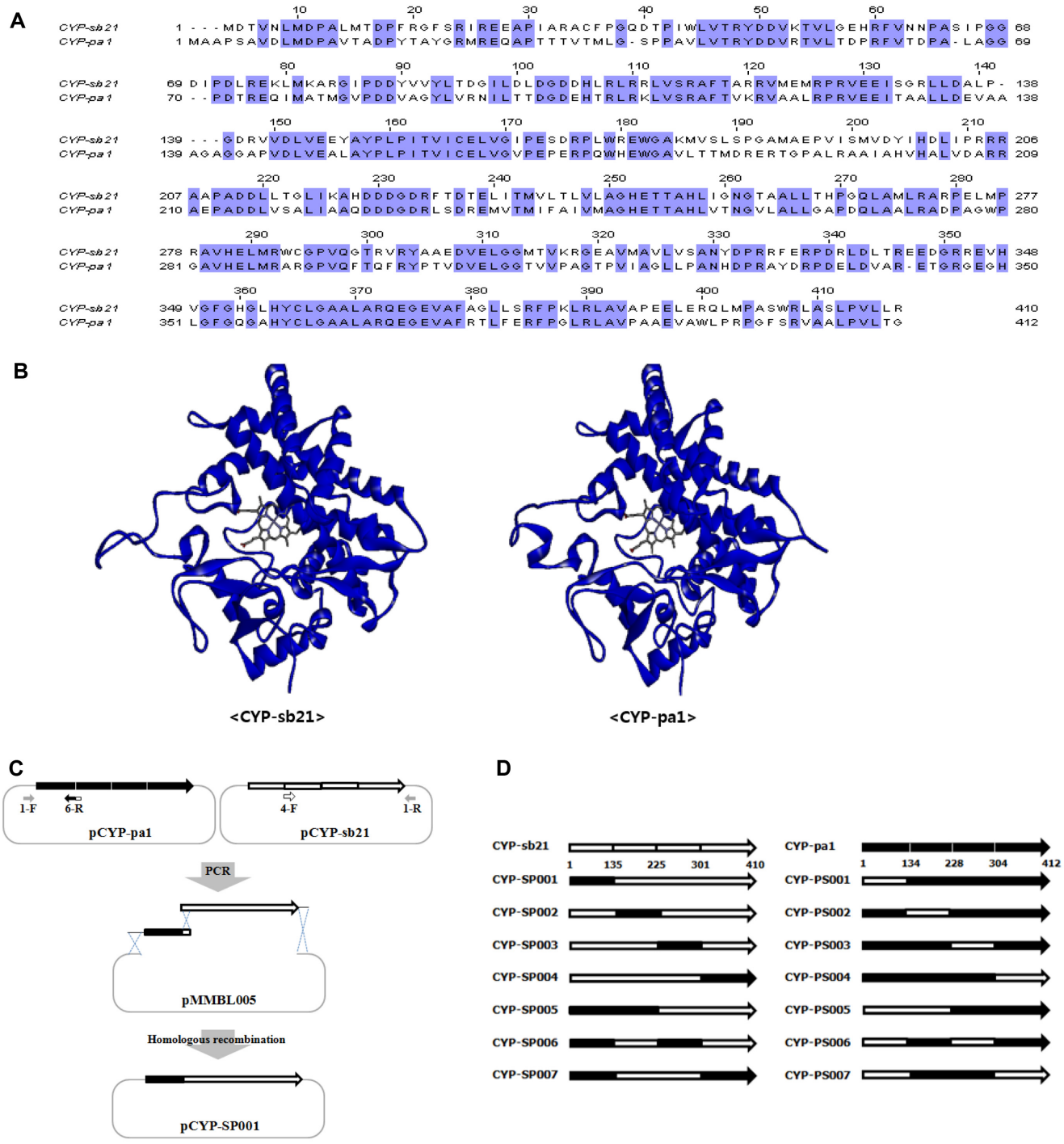

Fig. 1. Amino acid sequence alignment and domain swapping between CsA-specific CYPs.
(**A**) Amino acid sequence alignment between CYP-sb21 and CYP-pa1. (**B**) Predicted 3D structure of CYP-sb21 and CYP-pa1. (**C**) Construction of hybrid gene by In-Fusion cloning method. (**D**) Schematic representation of hybrid CYP gene constructs.

sb21 strains were too low to compare regio-selectivities (data not shown), *S. benihana* ΔCYP-sb21 was identified as an inappropriate host for hybrid CYP bioconversion analysis. In the case of recombinant *P. autotrophica* ΔCYP-pa1 strains, two recombinant strains (*P. autotrophica* ΔCYP-pa1/pCYP-SP007 and *P. autotrophica* ΔCYP-pa1/pCYP-PS002)

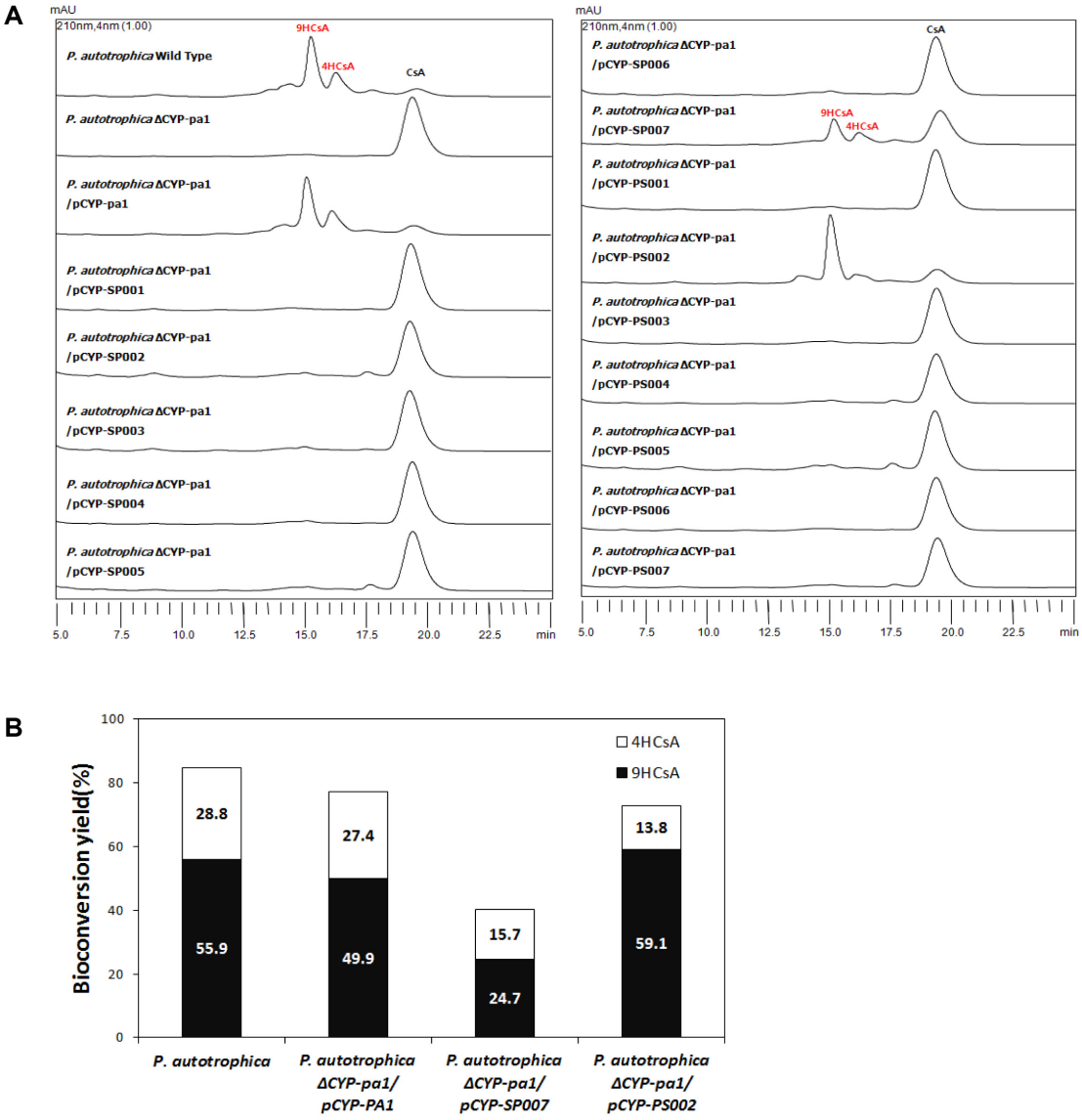

Fig. 2. CsA hydroxylation by hybrid CYPs.
(**A**) HPLC profiles of CsA bioconversion in *P. autotrophica* wild-type and *P. autotrophica* recombinant strains. (**B**) Bioconversion yields in *P. autotrophica* wild-type and *P. autotrophica* recombinant strains.

exhibited significant bioconversion activities (Fig. 2A). The 9HCsA/4HCsA ratio for *P. autotrophica* ΔCYP-pa1/pCYP-SP007 was 1.6, which was similar regio-selectivity as the *P. autotrophica* ΔCYP-pa1/pCYP-pa1 control (Fig. 2B). On the other hand, *P. autotrophica* ΔCYP-pa1/pCYP-pPS002 showed a 9HCsA/4HCsA ratio of 4.3, indicating stronger preference for 9HCsA over CYP-pa1 (Fig. 2B). This result suggests that the region critical for CsA regio-specific hydroxylation to 9HCsA is located in the 2nd domain of CYP-pa1 (135–228).

Identification of the Regio-Selectivity Region in the Second Domain

To determine which section of the 2nd domain (135–228) affects regio-selectivity, amino acid comparison was performed on the 2nd domains of CYP-sb21 and CYP-pa1. The two sections showing the largest differences were selected (Fig. 3A). We then produced an additional four hybrid CYP genes after altering the two sections of CYP-sb21 and CYP-pa1 and named them CYP-SP008, CYP-SP009, CYP-PS008, and CYP-PS009. To determine whether

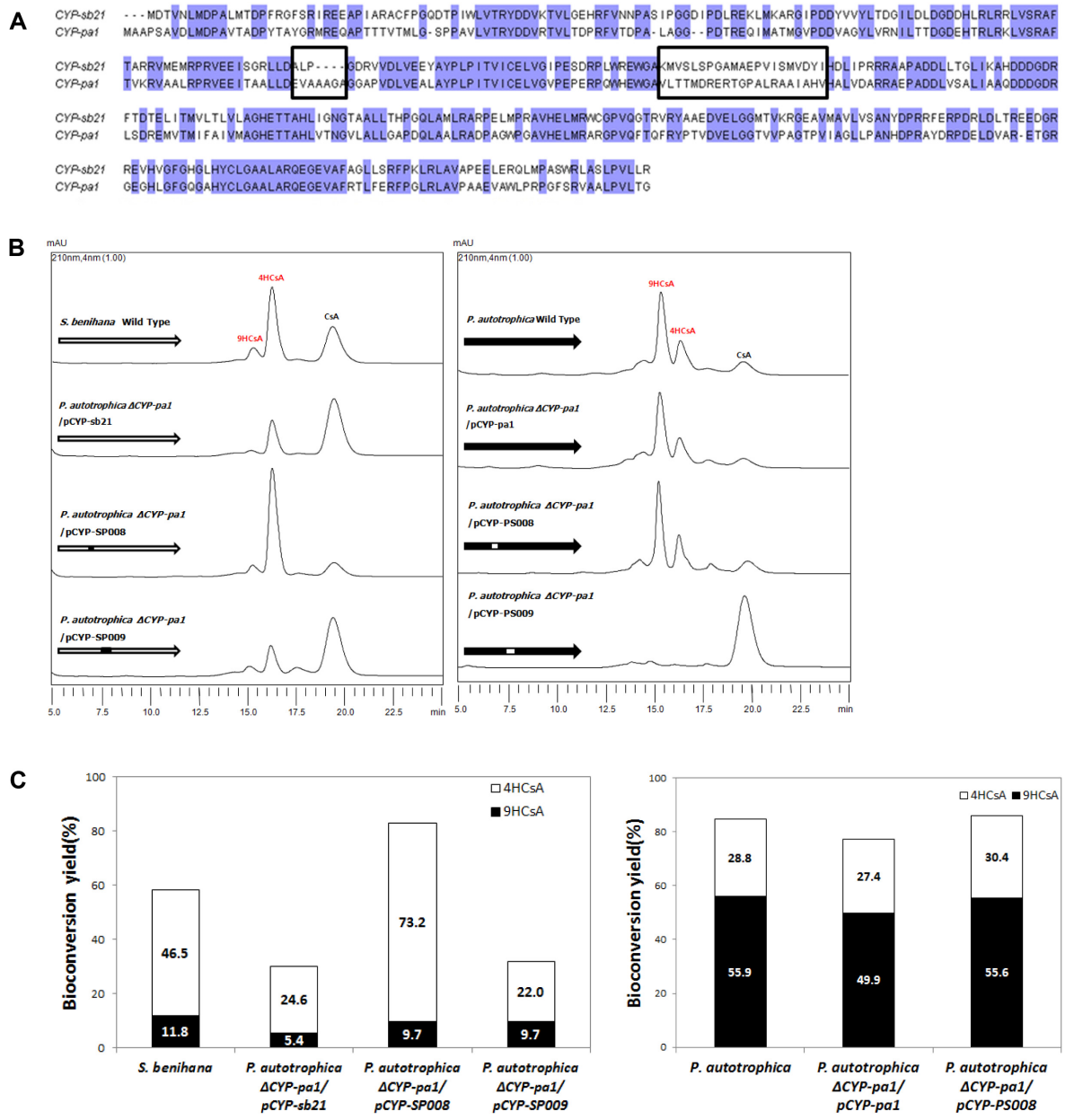

Fig. 3. Identification of the regio-selectivity region in the second domain.
(**A**) Amino acid sequence alignment between 2nd domains in CYP-sb21 and CYP-pa1. Swapping residues are indicated by boxes. (**B**) HPLC profile of CsA bioconversion in *P. autotrophica* wild-type and *P. autotrophica* recombinant strains. (**C**) Bioconversion yields in *P. autotrophica* wild-type and *P. autotrophica* recombinant strains.

or not CsA is hydroxylated by each hybrid CYP gene, the hybrid CYP gene construct was introduced into *P. autotrophica* ΔCYP-pa1, and bioconversion analysis was conducted. There was not much difference in bioconversion or regio-selectivity between *P. autotrophica* ΔCYP-pa1/pCYP-SP009 and *P. autotrophica* ΔCYP-pa1/pCYP-PS008 (Fig. 3B). On the other hand, *P. autotrophica* ΔCYP-pa1/pCYP-SP008 showed approximately 52% more bioconversion than *P. autotrophica* ΔCYP-pa1/pCYP-sb21, and the 4HCsA/9HCsA ratio was 7.5 (Fig. 3C). Compared with *S. benihana* wild-type, *P. autotrophica* ΔCYP-pa1/pCYP-SP008 showed approximately 27% more bioconversion of CsA to 4HCsA than 9HCsA (Fig. 3C), implying the 2nd domain of CYP-sb21 also induced conversion of CsA to 4HCsA (136-225 of CYP-sb21).

Discussion

The potential of hydroxylated CsA as a potential hair growth stimulator has been proven to be 100 times more effective than the existing product minoxidil, in the context of a mice skin graft experiment. The position of CsA hydroxylation is very important, since 4HCsA shows 10 times less immunosuppressive activity than 9HCsA (LG Household and Health Care. 2007. Korean Patent 1008652110000, LG Household and Health Care. 2008. Korean Patent 1006816700000), implying that CsA hydroxylation regio-selectivity might be important to the potential development of hydroxylated CsA as a hair growth stimulator with fewer side effects.

Here, we identified the domain of CYP that determines region-selectivity for CsA by the domain switch method. Using amino acid sequence comparison and 3D structure estimation, CYP-sb21 and CYP-pa1 were divided into four domains, which were then switched with each other. Fourteen mutant CYPs with switched domains were introduced into *S. benihana* ΔCYP-sb21 and *P. autotrophica* ΔCYP-pa1, and bioconversion experiments for CsA were conducted. In the bioconversion experiment, the 2nd domain of CYP-pa1 showed regio-selectivity in *P. autotrophica* ΔCYP-pa1/pCYP-PS002 with a 9HCsA/4HCsA ratio of 4.3. In other words, mutant CYP containing the 2nd domain of CYP-sb21 preferentially converted CsA to 9HCsA. To better identify the 2nd domain affecting regio-selectivity, amino acid sequence comparison was performed on the 2nd domains of CYP-sb21 and CYP-pa1. The 4HCsA/9HCsA ratio in *P. autotrophica* ΔCYP-pa1/pCYP-SP008 was 7.5, indicating a preference for 4HCsA over CYP-sb21 as well as 27% higher conversion yield than *S. benihana* wild type. These results imply that the 2nd domain of CsA-specific CYP plays a critical role in CsA regio-selectivity, thereby setting the stage for biotechnological application of CsA regio-selective hydroxylation.

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