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Identification of a Cyclosporine-Specific P450 Hydroxylase Gene through Targeted Cytochrome P450 Complement (CYPome) Disruption in *Sebekia benihana*

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It was previously proposed that regio-specific hydroxylation of an immunosuppressive cyclosporine (CsA) at the 4th *N*-methyl leucine is mediated by cytochrome P450 hydroxylase (CYP) in the rare actinomycete *Sebekia benihana*. This modification is thought to be the reason for the hair growth-promoting side effect without the immunosuppressive activity of CsA. Through *S. benihana* genome sequencing and *in silico* analysis, we identified the complete cytochrome P450 complement (CYPome) of *S. benihana*, including 21 CYPs and their electron transfer partners, consisting of 7 ferredoxins (FDs) and 4 ferredoxin reductases (FDRs). Using *Escherichia coli* conjugation-based *S. benihana* CYPome-targeted disruption, all of the identified CYP, FD, and FDR genes in *S. benihana* were individually inactivated. Among the 32 *S. benihana* exconjugant mutants tested, only a single *S. benihana* CYP mutant, ΔCYP-sb21, failed to exhibit CsA hydroxylation activity. The hydroxylation was restored by CYP-sb21 gene complementation. Since all *S. benihana* FD and FDR disruption mutants maintained CsA hydroxylation activity, it can be concluded that CYP-sb21, a new member of the bacterial CYP107 family, is the only essential component of the *in vivo* regio-specific CsA hydroxylation process in *S. benihana*. Moreover, expression of an extra copy of the CYP-sb21 gene increased CsA hydroxylation in wild-type *S. benihana* and an NADPH-enriched *Streptomyces coelicolor* mutant, by 2-fold and 1.5-fold, respectively. These results show for the first time that regio-specific hydroxylation of CsA is carried out by a specific P450 hydroxylase present in *S. benihana*, and they set the stage for the biotechnological application of regio-specific CsA hydroxylation through heterologous CYP-sb21 expression.

Bacterial cytochrome P450 hydroxylase (CYP) enzymes belong to a superfamily of heme-containing monooxygenases that catalyze numerous types of reactions, including biotransformation of natural compounds such as polyketides, fatty acids, steroids, and some environmental xenobiotic compounds (1, 2). The catalytic abilities of CYPs are highly attractive due to their superior regio- and stereo-selectivities, thereby introducing the possibility of various biotechnological applications, such as hydroxylation of structurally diverse natural as well as synthetic compounds by CYP-driven bioconversion processes (1). In general, the bacterial CYP is known to transfer one oxygen atom from a CYP-bound oxygen molecule along with one hydrogen atom from NAD(P)H, which is regenerated by the ferredoxin (FD)-ferredoxin reductase (FDR) electron transfer system, to its substrate (3).

Streptomycetes and the physiologically related actinomycetes are soil microorganisms that are well known for their ability to produce both pharmacologically important secondary metabolites as well as an abundance of various bioconversion enzymes, including CYPs (4). A rare actinomycete named *Sebekia benihana* was previously reported as an important microorganism for natural product bioconversion, since this strain demonstrates unique regio-specific hydroxylation abilities on several structurally diverse substrates, including cyclosporine (CsA) (2, 5–7). The cyclic undecapeptide CsA, one of the most valuable immunosuppressive drugs today, is typically produced nonribosomally by a multifunctional cyclosporine synthetase enzyme complex in the filamentous fungus *Tolypocladium niveum*. In addition to its immunosuppressive activity, CsA provokes several side effects, including hirsutism, a phenomenon of abnormal bodily hair growth. Treatment by intraperitoneal injection or topical application of CsA has been shown to favor the dystrophic anagen pathway as well as to offer protection against dystrophy and alopecia in mice (8–10). Previously, a CsA derivative, γ-hydroxy-*N*-methyl-L-Leu4-CsA (Fig. 1), was reported to have hair growth-promoting effects without the immunosuppressive activity characteristic of CsA (11). After screening of thousands of actinomycete strains for CsA-specific bioconversion capabilities, *S. benihana* was selected as the strain showing the highest regio-specific CsA hydroxylation activity at the 4th *N*-methyl leucine. This interesting observation led to the assumption that a unique regio-specific CsA hydroxylation process driven by a single or combination CYP system(s) is present in *S. benihana*. This novel system could also be applied for the production of a potentially valuable hair growth-stimulating compound.

Previously, six *S. benihana* CYP genes (tentatively named *S. benihana* CYP501 to -506) were identified through a PCR-driven search for conserved motifs present in bacterial CYPs, followed by their expression in a CsA-nonhydroxylating heterologous host (7, 12). Although CsA hydroxylation was barely induced by coexpression of *S. benihana* CYP506 with *Streptomyces coelicolor* FD in the heterologous system, it remained ambiguous whether or not *S. benihana* CYP506 indeed played a direct role in the CsA hydroxy-

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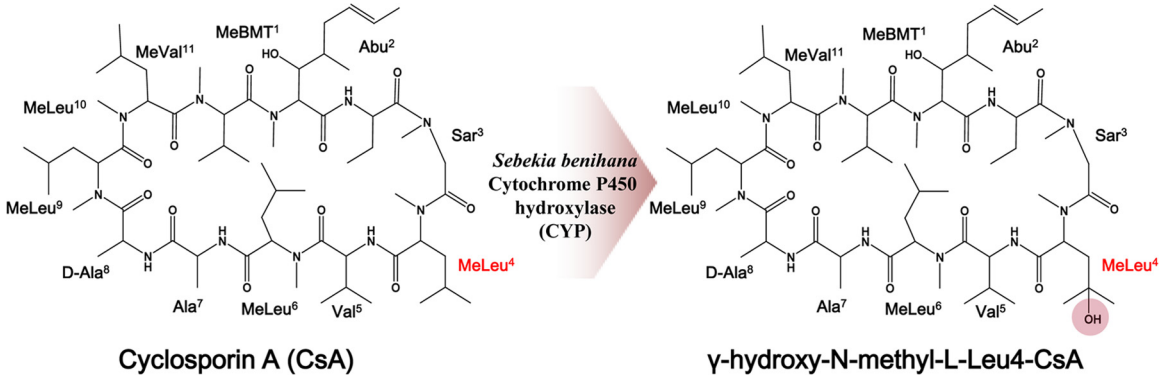

FIG 1 Biosynthetic conversion of CsA to γ-hydroxy-*N*-methyl-L-Leu4-CsA by *S. benihana*.

lation process, due to the lack of a genetic confirmation method for *S. benihana* (7, 12). Recently, however, we successfully established an *Escherichia coli* conjugation-based foreign gene transfer and expression system, as well as a targeted gene disruption protocol for *S. benihana* (13). Using this optimized *S. benihana* genetic manipulation system, we showed that *S. benihana* CYP506 was not the major CsA-specific hydroxylase in the CsA hydroxylation process (13). This indicated that regio-specific CsA hydroxylation might require another unique CYP and/or an FD-FDR system in *S. benihana*. Here we report the identification and characterization of the complete *S. benihana* cytochrome P450 complement (CYPome), including 21 CYPs along with their electron partners, consisting of 7 FDs and 4 FDRs, by whole-genome sequencing and *in silico* analysis, followed by genetic confirmation of a unique CYP responsible for regio-specific CsA hydroxylation, based on *S. benihana*-targeted CYPome disruption as well as functional expression of the target CYP gene in a CsA-nonhydroxylating *Streptomyces* heterologous host.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used for this study are given in Table 1. *Escherichia coli* DH5α was used as the cloning host. Plasmids were propagated in *E. coli* ET12567 in order to obtain unmethylated DNA for transformation into *S. benihana*. *E. coli* was grown in Luria-Bertani (LB) broth, maintained on LB agar medium at 37°C, and supplemented with appropriate antibiotics when needed. *S. benihana* (KCTC 9610), obtained from the Korean Collection for Type Cultures (KCTC; South Korea), was cultured on GSMY (0.7% glucose, 0.45% yeast extract, 0.5% malt extract, 1.0% soluble starch, and 0.005% calcium carbonate) at 28°C with constant shaking at 200 rpm for 3 days, followed by cell harvesting and total DNA isolation. *S. benihana* exconjugants were supplemented with apramycin (25 μg/ml) or hygromycin (25 μg/ml). The pMMBL005 vector was constructed through subcloning of the PermE* promoter region between EcoRI and BamHI sites, followed by replacement of the apramycin resistance gene with the hygromycin resistance gene in pSET152.

***S. benihana* genome sequencing for identification of the CYPome.** The draft genome sequence of *S. benihana* was obtained on a model 454 GS-FLX (Roche) system (total of 684,556 reads, with an average length of 417.7 bp) and by traditional whole-genome Sanger shotgun sequencing (total of 12,576 reads, with an average length of 702.1 bp), resulting in two genome libraries (insert sizes of ~2 kb and ~35 kb) generated by random shearing of genomic DNA. The sequence data were assembled using Newbler, the Phred/Phrap/Consed package, and in-house scripts. Protein-encoding genes were predicted using Glimmer 3.0 (19); tRNA and rRNA were identified using tRNAscan-SE (14) and RNAmmer (20), respectively. Functions of the predicted protein-encoding genes were annotated by comparisons with the UniRef90 (21), NCBI-NR (22), COG (23), and KEGG (24) databases.

***S. benihana* CYPome disruption and mutant complementation.** Mutant strains were constructed using a PCR-targeted gene disruption system according to the general method detailed by Gust et al. (17), with some modifications. An apramycin resistance gene-*oriT* cassette for replacement of the CYP-sb, FD-sb, or FDR-sb gene was amplified using pIJ773 as a template, along with disruption primers (see Table S1 in the supplemental material). The resultant PCR products were replaced by the CYP-sb, FD-sb, or FDR-sb gene in target cosmids, generating mutated cosmids pMJ001 to -035 in *E. coli* BW25113/pIJ790. Mutated cosmids were then transferred into *S. benihana* by conjugation via strain ET12567/pUZ8002, after which desired mutants (products of double crossover) were identified by screening for apramycin-resistant and kanamycin-sensitive colonies. The double-crossover exconjugants were selected according to a standard Apr^r Kan^s method (17), followed by confirmation of both wild-type *S. benihana* and *S. benihana* ΔCYP-sb, ΔFD-sb, or ΔFDR-sb genomic DNA by PCR. The three types of PCR primers used for confirmation of the double-crossover recombinants were the CYP-sb check F-R, FD-sb check F-R, and FDR-sb check F-R primers, in addition to *oriT* test primer F (5′-GAATTCAGCGTGACATCATTCTGTGG-3′), located in the Apr^r-*oriT* cassette. Oligonucleotide sequences and expected sizes of the disruption and check primers are listed in Table S1. For complementation of the ΔCYP-sb21 mutant, the recombinant integrative plasmid pMMBL302, containing the CYP-sb21 gene, was used along with pMMBL301, containing the CYP-sb13 (previously named CYP506) gene, as a negative control. Hygromycin was used for selection of recombinant *S. benihana* ΔCYP-sb21/pMMBL302 and *S. benihana* ΔCYP21-sb21/pMMBL301.

Construction of recombinant plasmids and conjugation into *S. benihana* and *S. coelicolor*. For functional overexpression and complementation of the CYP-sb21 gene in *S. benihana*, a 1.3-kb DNA fragment containing the entire CYP-sb21 gene was amplified by PCR using genomic DNA from *S. benihana* as a template, along with the following primers: forward primer, 5′-AGATCTCGGTCACGAGAGGCGACG-3′; and reverse primer, 5′-TCTAGACGCGACCTCTGTCCTCCG-3′. The PCR-amplified CYP-sb21 gene included the putative upstream ribosome binding site, start codon, and stop codon sequences. PCR was performed in a final volume of 20 μl containing 0.4 M (each) primers, a 0.25 mM concentration of each deoxynucleoside triphosphate (dNTP; Roche), 1 μg of extracted DNA, 1 U of *Ex Taq* polymerase (TaKaRa, Japan) in the recommended reaction buffer, and 10% dimethyl sulfoxide (DMSO). Amplifications were performed on a thermal cycler (Bio-Rad) 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 1% (wt/vol) agarose gel electrophoresis, purified using a DNA extraction kit (Cosmo, South Korea), and ligated into the pGEM-T Easy vector (TaKaRa, Japan). Complete

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid[a]	Characteristics	Source or reference
Strains		
Escherichia coli		
DH5α	Cloning host	KCTC
ET12567/pUZ8002	Strain for intergeneric conjugation; Km^r Cm^r	4
Sebekia benihana		
KCTC9610	Wild type	KCTC
ESK611	*cyp21*-complemented *S. benihana* ΔCYP21 with pMMBL301	This work
ESK612	*cyp21*-complemented *S. benihana* ΔCYP21 with pMMBL302	This work
ESK613	*cyp21*-overexpressing *S. benihana*	This work
Streptomyces coelicolor		
M145	Wild type	ATCC (BAA-471)
ESK614	CYP-sb21 gene-overexpressing *S. coelicolor* M145	This work
Δ*wblA*	*whiB*-like gene A (SCO3579)-deleted *S. coelicolor*	15
ΔSCO5426	6-Phosphofructokinase gene (SCO5426)-deleted *S. coelicolor*	16
Triple-knockout mutant	*S. coelicolor* Δ*wblA* ΔSCO1712 ΔSCO5426	16
ESK616	CYP-sb21 gene-overexpressing *S. coelicolor* ΔSCO5426	This work
ESK617	CYP-sb21 gene-overexpressing *S. coelicolor* Δ*wblA*	This work
ESK618	CYP-sb21 gene-overexpressing *S. coelicolor* Δ*wblA* ΔSCO1712 ΔSCO5426	This work
Recombinant plasmids		
T&A cloning vector	General cloning plasmid	Real Biotech Co., Taiwan
pIJ773	pBluescript vector carrying a gene disruption cassette containing an apramycin resistance gene and *oriT*	17
pMMBL005	pSET152 containing *ermE** and Hyg^r gene for *E. coli-Pseudonocardia autotrophica* conjugative vector	This work
pMMBL301	Vector for CYP-sb13 complementation; Hyg^r	13
pMMBL302	Vector for CYP-sb21 complementation and overexpression; Hyg^r	This work
pMMBL304	Vector for CYP-sb21 complementation (fused CYP-sb21 with CYP-sb13); Hyg^r	This work
pHSEV1	Shuttle vector containing the *tipA* promoter derived from pIJ4123	18
pMMBL312	Vector for heterologous expression of *cyp21* and P*tipA* promoter, Kan^r	This work

[a] See Materials and Methods for details on plasmid construction.

nucleotide sequencing was then performed by Genotech Korea, followed by subcloning into pMMBL302, a pSET152 derivative integrative plasmid expressing the strong constitutive promoter PermE* and a hygromycin resistance gene. The resultant pMMBL302 plasmid was introduced into *S. benihana*, *S. benihana* ΔCYP-sb21, and *S. coelicolor* M145 via conjugation, followed by integration via phiC31 attachment/integration and hygromycin selection.

HPLC analysis of CsA-specific hydroxylation. The selected *S. benihana* strains were first cultured in GSMY medium for 3 days, treated with 50 mg/liter of CsA as the hydroxylation substrate, and then further cultured for an additional 2 days for conversion. Samples for high-pressure liquid chromatography (HPLC) analysis were isolated both after addition of CsA and after the 2-day conversion period. Metabolites were analyzed by HPLC with a photo diode array detector and a symmetry C_{18} column in a two-buffer gradient system consisting of 25% methanol (buffer A) and 100% acetonitrile (buffer B). One cycle of buffer B gradient was programmed as follows: 40% for 4 min, 61% for 20 min, and 100% buffer B for 40 min, followed by 40% for 45 min. The column temperature was maintained at 60°C. CsA and its derivatives were monitored at 210 nm. The flow rate was 1.0 ml/min, and the injection volume was 20 μl.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *S. benihana* CYPs are KC208044 to KC208064.

RESULTS

***S. benihana* genome sequencing and bioinformatic analysis of the CYPome.** The draft genome sequence of *S. benihana* comprised 8,830,359 bases assembled into 2,754 contigs (>500 bp), and it had a GC content of 69.7%. Furthermore, there were 62 predicted tRNA sequences along with 11,464 protein coding sequences (CDSs) in the genome sequence. Specifically, the coding percentage was 73%, and 4,320 CDSs showed functional predictions. Using COG functional assignment, the majority of predicted proteins (47.2%) were classified into 23 COG categories (see Table S2 in the supplemental material). We identified a total of 25 distinct cytochrome P450 hydroxylase (CYP) genes from *S. benihana* (encoding CYP-sb1 to -24) (Table 2), followed by complete sequencing and *in silico* characterization. Four *S. benihana* CYP-sb genes (the CYP-sb5, CYP-sb14, CYP-sb18, and CYP-sb19 genes) initially predicted by the genome sequence scan were determined to be incomplete CYP genes.

The conserved CYP-sb structural core is formed by a four-helix bundle comprised of three parallel helices (D, L, and I) and one antiparallel helix, E, wherein the prosthetic heme group is confined between the distal I helix and the proximal L helix as well as bound to adjacent cystine residues of heme domains (25, 26). Table S3 in the supplemental material shows the motifs that most closely correspond to these conserved CYP expectations. All 21 CYP-sb enzymes of *S. benihana* possessed a conserved I helix containing the signature amino acid sequence (A/G)GXX(A/T). Threonine in the I helix, which was highly conserved among all

TABLE 2 CYPome of *S. benihana*, with closest homologues of the CYPs

CYPome member	No. of aa	Closest match in the database[a]			
		Protein name	Species	% identity/ % similarity	GenBank accession no.
CYP-sb1	408	MoxA	*Nonomuraea recticatena*	86/92	BAE78751
CYP-sb2	228	MoxA	*Nonomuraea recticatena*	93/97	BAE78751
CYP-sb3-1	397	CYP107L14	*Streptomyces pristinaespiralis* ATCC 25486	51/64	ZP_06907823
CYP-sb3-2	397	CYP107L14	*Streptomyces virginiae*	50/65	ABR68807
CYP-sb4	446	CYP107B1	*Stigmatella aurantiaca* DW4/3-1	45/58	ZP_01459433
CYP-sb6	387	CYP105C1	*Saccharothrix espanaensis* DSM 44229	57/71	CCH28866
CYP-sb7	405	CYP105C1	*Streptomyces cattleya* NRRL 8057	59/71	YP_004920090
CYP-sb8	394	CYP125	*Rhodococcus opacus* PD630	58/71	EHI44531
CYP-sb9	416	CYP179A1	*Streptomyces avermitilis* MA-4680	61/72	NP_823237
CYP-sb10	408	CYP107B	*Streptomyces cattleya* NRRL 8057	37/52	YP_004915121
CYP-sb11	406	CYP107B1	*Nocardia cyriacigeorgica* GUH-2	73/88	YP_005265788
CYP-sb12	386	CYP107P2	*Streptomyces avermitilis* MA-4680	62/75	NP_825716
CYP-sb13	402	CYP105C1	*Streptomyces cattleya* NRRL 8057	72/82	YP_004920090
CYP-sb15	456	CYP262A1	*Streptosporangium roseum* DSM 43021	51/70	ACZ86135
CYP-sb16	408	CYP105L	*Streptomyces virginiae*	59/68	ABR68806
CYP-sb17	395	CYP105C1	*Saccharothrix espanaensis* DSM 44229	49/63	CCH35687
CYP-sb20	383	CYP105C1	*Amycolatopsis* sp. ATCC 39116	54/65	ZP_10055035
CYP-sb21	410	CYP107Z3	*Streptomyces* sp. IHS-0435	61/77	AAT45266
CYP-sb22	938	CYP102D1	*Streptomyces avermitilis* MA-4680	60/75	NP_821750
CYP-sb23	357	MoxA	*Nonomuraea recticatena*	98/99	BAE78751
CYP-sb24	320	CYP107B1	*Streptomyces cattleya* NRRL 8057	42/56	YP_004915121
FD-sb1	72	Ferredoxin	*Nonomuraea recticatena*	95/100	BAE78752
FD-sb2	64	Ferredoxin	*Streptosporangium roseum* DSM 43021	64/77	YP_003341724
FD-sb4	66	Ferredoxin	*Nonomuraea recticatena*	100/100	BAE78752
FD-sb5	44	Ferredoxin	*Saccharomonospora glauca* K62	73/83	ZP_10013511
FD-sb6	127	Ferredoxin	*Gordonia rhizosphera* NBRC 16068	75/87	ZP_10948649
FD-sb8	146	Ferredoxin	*Streptosporangium roseum* DSM 43021	73/81	YP_003339036
FD-sb10	73	Ferredoxin	*Streptosporangium roseum* DSM 43021	60/81	YP_003341724
FDR-sb1	63	Reductase	*Frankia* sp. EuI1c	53/62	YP_004014432
FDR-sb2	67	Reductase	*Frankia* sp. CN3	58/67	ZP_09167510
FDR-sb3	415	Reductase	*Streptosporangium roseum* DSM 43021	82/88	YP_003337828
FDR-sb4	289	Reductase	*Streptomyces sviceus* ATCC 29083	63/72	ZP_06915399

[a] Based on database search of the NCBI website (www.ncbi.nlm.nih.gov/BLAST/).

CYP-sb enzymes except for CYP-sb12, is believed to be involved in oxygen activation (27, 28). Similarly, glutamic acid and arginine in the EXXR motif of the K helix were highly conserved in all the *S. benihana* CYP-sb enzymes. Both of these residues help to form a set of salt bridge interactions that participate in the formation of the final CYP tertiary structure (29). Moreover, the heme-binding signature amino acid sequence FXXGXXXCXG was conserved among all 21 CYP-sb enzymes (see Table S3). The absolutely conserved cysteine residue is believed to participate in the formation of two hydrogen bonds with the neighboring backbone amide and serves as the fifth axial ligand of the heme iron. Furthermore, the sulfur ligand, a thiolate, is responsible for the characteristic 450-nm Soret peak of the ferrous CO complex that is the namesake of CYPs (30).

In general, CYPs are grouped into different families when they exhibit <40% amino acid sequence identity, and the two most abundant *Streptomyces* CYP families are CYP105 and CYP107. While most CYP105s are apparently promiscuous in their range of catalytic activities, many CYP107s are found to be involved in antibiotic biosynthetic pathways (31, 32). Using an *in silico* sequence similarity search, nine *S. benihana* CYPs (CYP-sb1, CYP-sb2, CYP-sb6, CYP-sb7, CYP-sb13, CYP-sb16, CYP-sb17, CYP-sb20, and CYP-sb23) were identified as members of the bacterial CYP105 family. Recently, several enzymes belonging to the CYP105 family were characterized as playing crucial roles in xenobiotic degradation as well as antibiotic biosynthesis (30–33). CYP-sb13, previously named an *S. benihana* CYP506, was proposed to play a limited role in the CsA hydroxylation process, probably through indirect interactions with other CYP-FD-FDR systems (13). Eight other *S. benihana* CYPs (CYP-sb3-1, CYP-sb3-2, CYP-sb4, CYP-sb10, CYP-sb11, CYP-sb12, CYP-sb21, and CYP-sb24) belong to the bacterial CYP107 family (34–36). The remaining four *S. benihana* CYPs (CYP-sb8, CYP-sb9, CYP-sb15, and CYP-sb22) each belong to different bacterial CYP families. Specifically, CYP-sb8 and CYP-sb15, which were both identified from *Sorangium cellulosum* So ce56, were shown to belong to the CYP125 and CYP262 families, respectively (30). CYP-sb9 belongs to the CYP179A1 family, showing 61% amino acid identity with *Streptomyces avermitilis* SAV_2061 (37). Interestingly, CYP-sb22, which belongs to the bacterial CYP102D1 family, shows 60% amino acid identity with the cytochrome P450/NADPH-ferrihemoprotein reductase from *S. avermitilis* (38). This bifunctional protein is fused at its N-terminal cytochrome P450 with cytochrome P450 oxidoreductase (CYPOR). NADPH cytochrome P450 reductase serves as an electron donor in several oxygenase sys-

tems, as well as being a component of nitric oxide synthases and methionine synthase reductases. CYPOR transfers two electrons from NADPH to the heme of cytochrome P450 via flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). This CYP architecture has also been observed for CYP102 homologues in *Bacillus subtilis* and *S. coelicolor* A3(2) (39, 40).

Most bacterial CYPs associate with electron-recycling partners such as ferredoxin (FD) and ferredoxin reductase (FDR) in a complex for the functional expression of their catalytic activities (39). Here we identified seven putative FD-sb and four FDR-sb genes present in *S. benihana*, which implies that the functional activities displayed by the 21 *S. benihana* CYP-sb enzymes are the result of different combinations of FD-sb and FDR-sb partner proteins. Interestingly, the CYP-sb2, CYP-sb6, CYP-sb13, CYP-sb23, and CYP-sb24 genes were immediately followed by the FD-sb1, FD-sb2, FD-sb10, FD-sb4, and FD-sb5 genes, respectively, in *S. benihana* in terms of chromosomal location, suggesting that these electron transfer systems are associated with their own specific CYPs as natural redox partners. A unique arrangement was identified for the CYP-sb22 gene, as it encoded a single polypeptide consisting of the CYP fused to an FDR at its C terminus for shuttling of electrons to the CYP (41). Phylogenetic relatedness among *S. benihana* CYPs-sb, FDs-sb, and FDRs-sb was determined (see Fig. S1 in the supplemental material).

Targeted CYPome disruption in *S. benihana*. It was previously demonstrated that *S. benihana* efficiently hydroxylates CsA at the 4th *N*-methyl leucine in a regio-specific manner (11). To identify the target gene responsible for this regio-specific CsA hydroxylation process in *S. benihana*, inactivation of each CYP-sb gene was performed using a previously optimized PCR-targeted gene disruption system (see Materials and Methods). An apramycin resistance (Apr^r)-*oriT* cassette was added to the cosmids used for whole-genome sequencing in order to generate pMJ001 to -024, which were then introduced into *S. benihana* by conjugative gene transfer (Fig. 2A). Construction of CYP mutants (*S. benihana* ΔCYP-sb mutants) generated by PCR-targeted gene disruption was confirmed by PCR using the CYP-sb check primers. The expected PCR-amplified bands were observed for genomic DNA samples isolated from *S. benihana* as well as from *S. benihana* ΔCYP-sb (Fig. 2A), implying that each CYP gene was specifically disrupted as expected. Fermentation broths of both *S. benihana* and the independent *S. benihana* ΔCYP mutants grown under optimal conditions for CsA hydroxylation were extracted using ethyl acetate, followed by HPLC quantification (Fig. 2C). In comparing CsA hydroxylation yields between the wild type and the ΔCYP-sb mutants, the *S. benihana* ΔCYP-sb1, ΔCYP-sb2, ΔCYP-sb3-1, ΔCYP-sb3-2, ΔCYP-sb11, ΔCYP-sb20, ΔCYP-sb23, and ΔCYP-sb24 strains showed similar or slightly increased hydroxylation yields compared to the wild-type strain, implying that these genes have minor effects on the CsA hydroxylation process. On the other hand, hydroxylation yields of more than half of all ΔCYP-sb mutants, including the ΔCYP-sb4, ΔCYP-sb6, ΔCYP-sb7, ΔCYP-sb8, ΔCYP-sb9, ΔCYP-sb10, ΔCYP-sb12, ΔCYP-sb13, ΔCYP-sb15, ΔCYP-sb16, ΔCYP-sb17, and ΔCYP-sb22 strains, were decreased by 25% to 50% (Fig. 2C).

Identification and characterization of the CYP-sb21 gene in *S. benihana*. With our CYP-sb21 gene disruption strain, in which the ΔCYP-sb21 mutation completely abolished regio-specific CsA hydroxylation in *S. benihana*, there was strong evidence that CYP-sb21 is the key enzyme involved in regio-specific CsA hydroxylation (Fig. 2C). To further prove that inactivation of the CYP-sb21 gene was indeed responsible for CsA hydroxylation, we performed genetic complementation of *S. benihana* ΔCYP-sb21. The coding sequence of the CYP-sb21 gene was cloned into a pMMBL005 *Streptomyces* expression vector harboring the constitutive promoter PermE* and the hygromycin resistance gene (named pMMBL302). The negative control was an expression vector containing the CYP-sb13 gene (named pMMBL301). Hygromycin was used for the selection of recombinant strains bearing the complementation plasmid. Plasmids were independently conjugated to *S. benihana* ΔCYP-sb21 and integrated into the chromosome via the phiC31 attachment/integration mechanism. The *S. benihana* ΔCYP-sb21/pMMBL301 and *S. benihana* ΔCYP-sb21/pMMBL302 strains were confirmed by PCR analyses, after which they were designated *S. benihana* ESK611 and ESK612, respectively. As expected, HPLC analysis confirmed that regio-specific CsA hydroxylation activity driven by CYP-sb21 was restored to a significant level (approximately 67%) in the *S. benihana* ESK612 strain, while no CsA hydroxylation was observed in the *S. benihana* ESK611 strain (Fig. 3A and B). Moreover, a wild-type *S. benihana* strain containing an extra copy of the CYP-sb21 gene via chromosomal integration of pMMBL302 (named *S. benihana* ESK613) led to an approximately 2-fold increase in the CsA hydroxylation yield (Fig. 3A and B), which implies that CYP-sb21 is indeed the target CYP responsible for regio-specific hydroxylation of CsA in *S. benihana*.

Identification of electron transfer partners of CYP-sb21 for CsA hydroxylation. In general, CYPs require an electron transfer system for oxygen activation and substrate conversion (42). CYPs are known to transfer one oxygen atom from a CYP-bound oxygen molecule along with one hydrogen atom from NAD(P)H, which is regenerated by the FD-FDR electron transfer system (3). Based on the above results, we speculated that CYP-sb21 from *S. benihana* could also utilize the FD and FDR partners found in *S. coelicolor*. It has been reported that CYPs cooperate with a variety of heterologous electron transfer proteins (43). Nonetheless, specific CYPs demonstrate higher activities when they interact with specific FDs or FDRs (44). Similar to the case for FDs and CYPs, specific FDRs are known to be better electron donors for specific FDs than for others (45). Therefore, the electron flow and concomitant activities of CYP-sb21 could be increased by its specific FD and FDR partners in *S. benihana*. Since the CYP-sb21 gene was not clustered with its specific FD- and FDR-encoding genes (CYP-sb21-specific FD-sb and FDR-sb genes, respectively) in *S. benihana*, inactivation of the FD-sb and FDR-sb genes was performed using the same PCR-targeted gene disruption system used for CYP-sb disruption. The modified cosmids, pMJ025 to -032 for FD-sb disruption and pMJ033 to -036 for FDR-sb disruption, were constructed and introduced into *S. benihana* by conjugative gene transfer. Following this, the FD-sb and FDR-sb genes were replaced with the Apr^r-*oriT* cassette, generating mutants named *S. benihana* ΔFD-sb and *S. benihana* ΔFDR-sb (Fig. 2A and B). Although most *S. benihana* ΔFD-sb and ΔFDR-sb mutants showed similar CsA-specific hydroxylation yields to those of the wild type, *S. benihana* ΔFD-sb8 and ΔFDR-sb3 exhibited noticeably reduced CsA hydroxylation activities (Fig. 2C). These results imply that FD-sb8 and FDR-sb3 might not be the only partners for CYP-sb21, although they may play major roles in regio-specific CsA hydroxylation, along with CYP-sb21, in *S. benihana*. It is possible

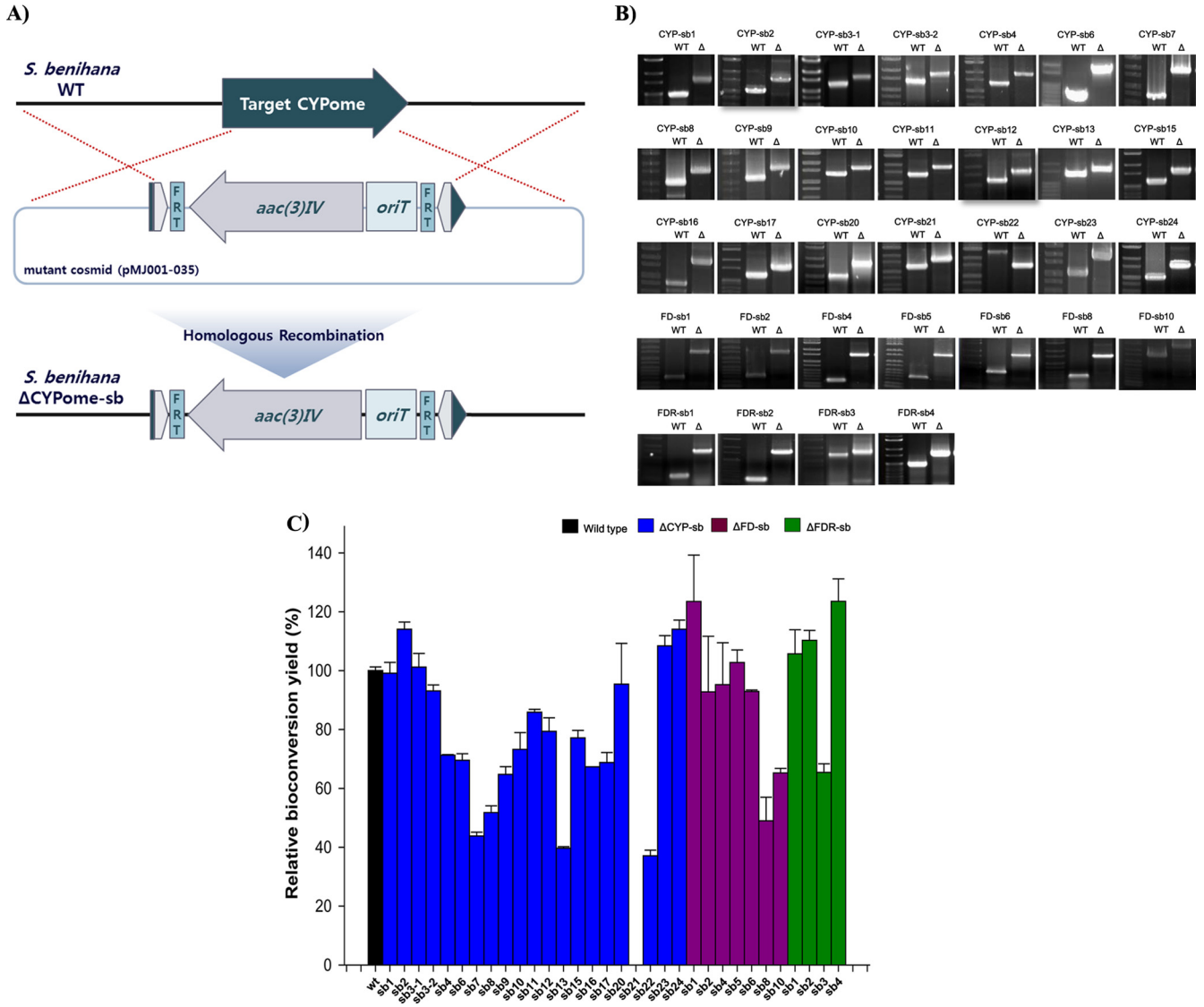

FIG 2 (A) Schematic representation of PCR-targeted CYPome gene replacement disruption. (B) Confirmation of the constructed *S. benihana* CYPome mutants by PCR analysis. The expected sizes of PCR products from the wild-type (WT) and ΔCYP-sb, ΔFD-sb, and ΔFDR-sb mutant (Δ) strains, using check F and check R primers, are listed in Table S1 in the supplemental material. The DNA size marker in the left lane is a 1-kb DNA ladder from Cosmo Genetech, South Korea, and represents bands of 3.0, 2.0, 1.5, 1.0, 0.8, and 0.6 kb, from top to bottom. (C) Relative conversion yields for CsA hydroxylation in *S. benihana* wild-type and ΔCYP-sb, ΔFD-sb, and ΔFDR-sb mutant strains. The error bars show the standard deviations of the means for three determinations.

that for *Streptomyces* species in general, combinations of relatively small numbers of FDs and FDRs are able to interact with and support all of the P450s in each species.

Functional expression of CYP-sb21 gene in a CsA-nonhydroxylating heterologous host. To maximize the genetic potential of CYP as a valuable industrial biocatalyst, we developed an efficient heterologous system for the expression of *S. benihana* CYP-sb21. We used *S. coelicolor* M145 as an expression host because of 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 was unable to carry out either CsA hydroxylation or any other CsA modification.

The coding sequence of the CYP-sb21 gene was cloned into a *Streptomyces* expression vector called pHSEV1 (named pMMBL312), which harbors the inducible P*tipA* promoter, as described in Materials and Methods (18). The pMMBL312 construct was transformed into *S. coelicolor* M145 through polyethylene glycol (PEG)-assisted protoplast transformation, followed by kanamycin selection. The resulting transformant was then confirmed to be the correct recombinant strain by PCR analysis and was named *S. coelicolor* ESK614. As expected, hydroxylation at the 4th *N*-methyl leucine of CsA was detected in an *S. coelicolor* ESK614 culture grown in CsA-containing broth as described in Materials and Methods (Fig. 3C and D).

To further improve CsA hydroxylation yields in a heterologous system, we tested several regulation-modified *S. coelicolor* mutants. Previously, we isolated two novel downregulatory genes involved in secondary metabolite biosynthesis (*wblA*, a WhiB-like

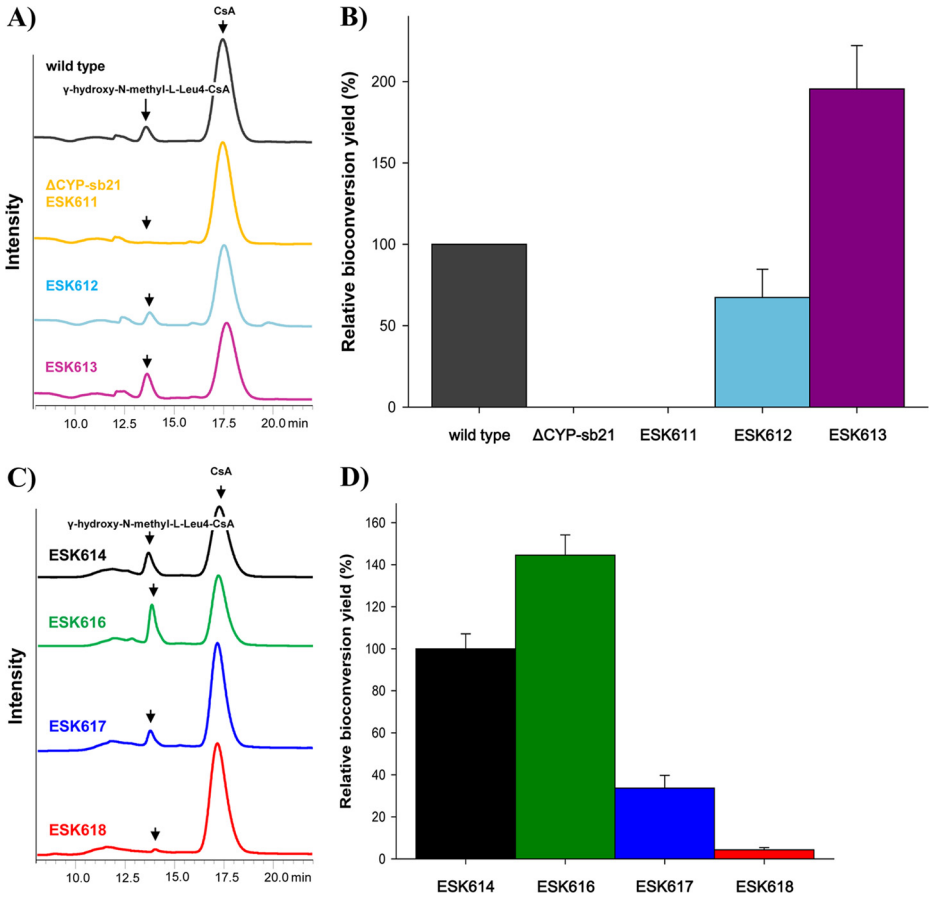

FIG 3 (A) HPLC chromatograms of CsA hydroxylation. (B) Comparison of relative CsA hydroxylation yields (%) in *S. benihana* wild type, *S. benihana* ΔCYP-sb21, *S. benihana* ESK611, *S. benihana* ESK612, and *S. benihana* ESK613. The error bars show the standard deviations of the means for three determinations. (C) HPLC chromatograms of CsA hydroxylation. (D) Comparison of relative CsA hydroxylation yields (%) in *S. coelicolor* ESK614, *S. coelicolor* ESK616, *S. coelicolor* ESK617, and *S. coelicolor* ESK618. The error bars show the standard deviations of the means for three determinations.

protein A gene, and SCO1712, a *tetR* family transcriptional regulatory gene) by using comparative transcriptome analysis (3, 24). Sequential targeted gene disruption of these two regulatory genes as well as SCO5426, a flux-controlling gene, resulted in a triple-knockout *S. coelicolor* mutant displaying significantly increased production of the polyketide antibiotic actinorhodin (15, 16, 46). To determine whether or not deletion of these regulatory genes could affect CsA hydroxylation activity in *S. coelicolor*, we introduced pMMBL312 into *S. coelicolor* mutants, generating *S. coelicolor* M145 ΔSCO5426, *S. coelicolor* M145 Δ*wblA*, and *S. coelicolor* M145 Δ*wblA* ΔSCO1712 ΔSCO5426 (designated ESK616, ESK617, and ESK618, respectively). Compared with the *S. coelicolor* M145 wild-type strain, both *S. coelicolor* ESK617 and ESK618 showed reduced hydroxylation yields (33% and 4%, respectively) (Fig. 3C and D), suggesting that deletion of the downregulatory genes involved in secondary metabolite biosynthesis did not stimulate CsA hydroxylation. Interestingly, however, ESK616 exhibited an approximately 1.5-fold increase in CsA hydroxylation (Fig. 3C and D), which implies that the *S. coelicolor* M145 ΔSCO5426 mutant strain is a better heterologous host for CsA hydroxylation. Recently, disruption of SCO5426, one of three 6-phosphofructokinase genes, was reported to enhance precursor carbon flux as well as NADPH supply through activation of the pentose phosphate pathway, resulting in significantly enhanced actinorhodin polyketide production in *S. coelicolor* (47). Therefore, these results suggest that the increased NADPH supply due to SCO5426 disruption might stimulate electron flow to the CYP-sb21 system, thereby enhancing the CsA hydroxylation yield.

DISCUSSION

CsA is one of the most widely prescribed immunosuppressive drugs for inhibiting graft rejection after organ transplantation. However, a major side effect of this potent immunosuppressive agent is the stimulation of hirsutism, a phenomenon of abnormal bodily hair growth. This observation sparked the hypothesis that a regio-specific modification of CsA could alter its bioactive spectrum, leading to dissociation of these two activities of CsA. Interestingly, it was reported that voclosporin A (VsA), a slightly modified CsA derivative containing only one additional methyl group, at the 1st amino acid position, enhances binding of the VsA-cyclophilin complex to calcineurin, resulting in increased potency and a more consistent pharmacokinetic-pharmacodynamic relationship than that with CsA (48). As a matter of fact, a group of scientists identified a rare actinomycete, *S. benihana*, which has reduced intrinsic immunosuppressive activity and hair growth-stimulating effects and mediates regio-specific hydroxylation of CsA at the 4th *N*-methyl leucine (30). Unfortunately, the bioconversion efficiency was low and difficult to improve in a less-char-

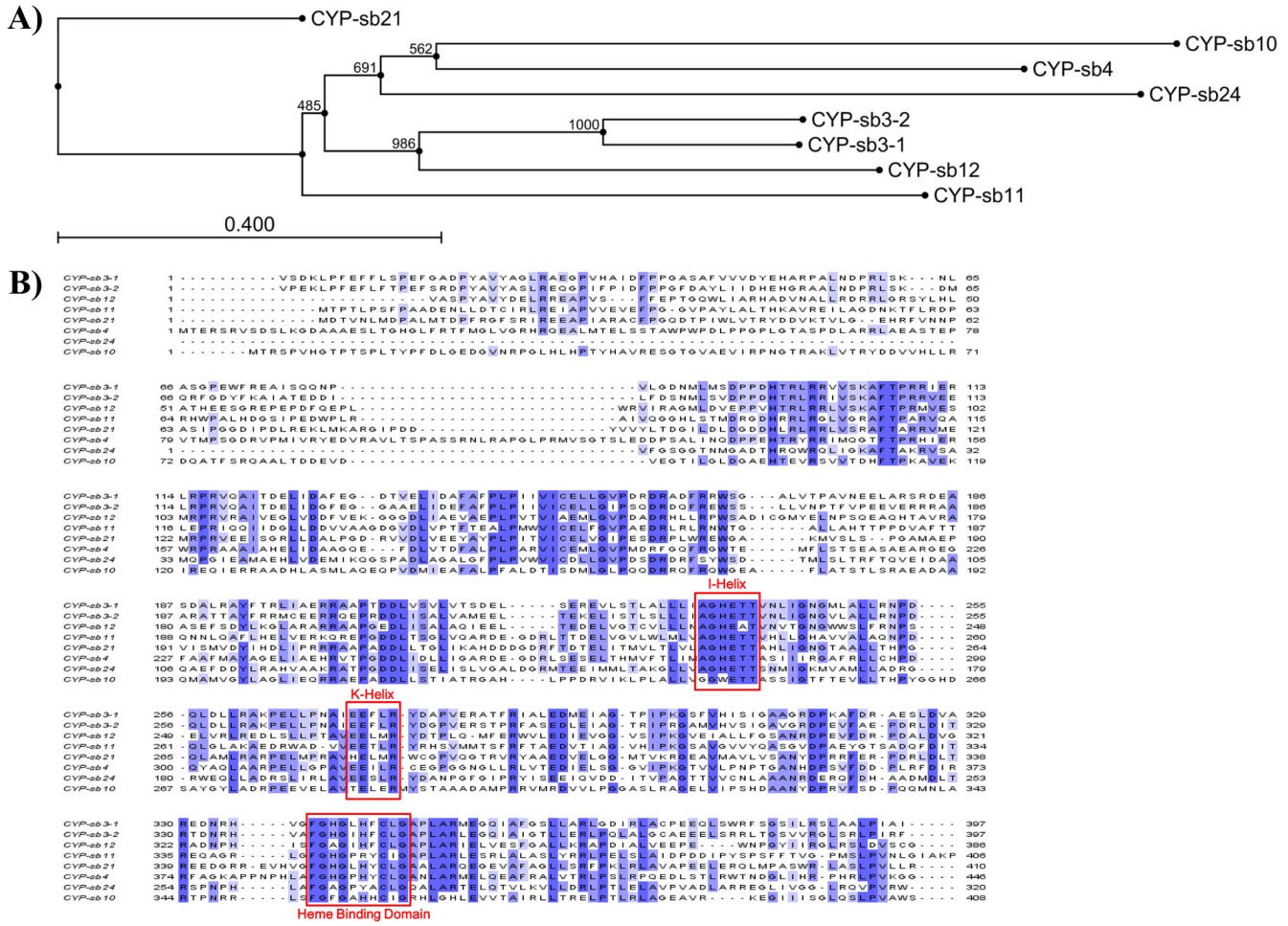

FIG 4 (A) Phylogenetic tree of *Sebekia benihana* cytochrome P450s belonging to the CYP107 family. The alignment was done with a 10-gap setting and 1-gap extension, with slow (very accurate) alignment input in a CLC Workbench. The tree was constructed by the neighbor-joining algorithm, with bootstrap analysis of 1,000 replicates. Bootstrap values are shown at branch nodes. The scale bar represents 0.4 amino acid substitution per amino acid. (B) Clustal W2 multiple-sequence amino acid alignment of CYP-sb21 and other CYPs-sb belonging to the CYP107 family. The multiple-sequence alignment was imported into Jalview for display. Bold and shaded cells indicate >50% amino acid identity and similarity among all sequences, respectively.

acterized bacterium such as *S. benihana*, especially for large-scale biotechnological application. Therefore, we established a targeted gene disruption system in *S. benihana* and searched for a unique CYP gene for regio-specific CsA hydroxylation to characterize its biological novelty as well as to generate an efficient heterologous expression system.

Using draft genome sequence analysis, we identified 21 CYP, 7 FD, and 4 FDR genes from *S. benihana*, followed by *in silico* characterization. *S. benihana* CYPome-disrupted mutant library screening showed that CsA hydroxylation completely disappeared only in the *S. benihana* ΔCYP-sb21 mutant strain, and it was restored by complementation in *trans* of the only CYP-sb21 gene. This provides strong evidence that CYP-sb21 is indeed the key enzyme involved in regio-specific CsA hydroxylation. As further confirmation, expression of an extra copy of the CYP-sb21 gene increased CsA hydroxylation in wild-type *S. benihana* as well as a CsA-nonhydroxylating heterologous host, *S. coelicolor*, suggesting that CYP-sb21 is necessary and sufficient for *in vivo* CsA hydroxylation.

Characterization of *S. benihana* CYP-sb21 via database-assisted *in silico* analysis revealed that it showed 61% amino acid identity to the cytochrome P450 monooxygenase CYP107z3 from *Streptomyces* sp. HIS-0435 and 66% amino acid identity to the cytochrome P450 from *Thermobispora bispora* DSM 43833. *S. benihana* CYP-sb21 harbors a strongly conserved threonine in its I helix that is reportedly involved in oxygen binding (49). It also features the E/DXXR motif in its putative K helix, along with a pocket with an invariable cysteine in the β-bulge preceding the L helix, both of which are involved in heme binding (Fig. 4B) (50, 51). In the conserved motif of CYP-sb21, there were only two amino acid differences from the L helix in CYP107z3 (Ema7), which is the enzyme responsible for oxidation of avermectin to 4″-oxo-avermectin in a regio-selective manner (38). Interestingly, CYP-sb21 was found to be related most distantly to other CYP107 family members in *S. benihana* (Fig. 4A).

Many CYPs are able to incorporate a single oxygen atom into a large number of biological substrates of various sizes and shapes with high degrees of stereo- and regio-specificity. Determining the structural and mechanistic basis of this versatility has been the focus of numerous structural investigations into CYPs (45, 52,

53). For example, three-dimensional structures of CYP107L1 from *Streptomyces venezuelae*, also named PikC, in complex with YC-17 and narbomycin, have been determined by X-ray crystallography (53). It was revealed that there are two modes of binding between the desosamine substituent of these antibiotics and the active site of PikC, which may explain the flexibility of PikC with respect to macrolactone ring substrates. The catalytic triad, consisting of residues D50, E85, and E94, is important for substrate recognition. Although CYP-sb21 also carries the similar acidic amino acid residues D37, R81, and D97 in corresponding positions, the relationship between catalysis and substrate specificity during CsA hydroxylation in *S. benihana* needs to be pursued further.

Bacterial P450s, which belong to the class I P450 system, are known to use a variety of electron transfer systems, in which electrons are always transferred from NAD(P)H. Various bacterial CYPs accept electrons from FDs, which receive them from FDRs (39). When the *S. coelicolor* ΔSCO5426 strain was used as an expression host for the CYP-sb21 gene, the CsA bioconversion yield was increased 1.5-fold compared to that of the *S. coelicolor* M145 wild type. These results indicate that *S. benihana* CYP-sb21 is capable of forming an enzyme complex with the FDs and FDRs from *S. coelicolor* and that the increased supply of NADPH due to SCO5426 disruption enhances electron flow to CYP-sb21 for improvement of CsA hydroxylation. The streptomycete species examined thus far contain several FD and FDR proteins, and attempts to discern the relevant electron transfer pathways have not been successful. As mentioned above, bacterial CYPs are known to use a variety of electron transfer mechanisms. For example, there are 18 CYPs, 6 FDs, and 4 FDRs in *S. coelicolor* A3(2), providing 24 possible pathways for electron transfer to each CYP. Chun et al. (54) established the primary electron transfer pathway used by CYP105D5-catalyzed fatty acid hydroxylation in *S. coelicolor* A3(2). Specific CYPs demonstrate higher activities upon interaction with specific FDs and FDRs, which are known to be better electron donors for certain FDs than for others (13, 44). To verify the electron transport system for CYP-sb21 in CsA hydroxylation, FD- or FDR-disrupted *S. benihana* mutants were constructed. Most of them showed similar CsA hydroxylation yields despite the disruption of each FD or FDR. The most notable decrease in CsA hydroxylation yield was observed with the ΔFD-sb8 and ΔFDR-sb3 mutants, implying that the respective proteins are the preferred *in vivo* partners of CYP-sb21 in regio-specific CsA hydroxylation in *S. benihana*. The amino acid composition of *S. benihana* FDR-sb3 is similar to those of flavoprotein reductases of other mono- and dioxygenase systems (55). As is the case with most of these proteins, the $NAD(P)^+$-binding domain is present at the N terminus of FDR-sb3, with the consensus sequence GXGXXA (residues 128 to 133). As there is an alanine instead of the third glycine, which is thought to be a major determinant of the $NADP^+$ versus NAD^+ specificity of enzymes, FDR-sb3 is specific for NADPH (43).

Although *S. benihana* CYP-sb21 could be expressed successfully in both *E. coli* and *S. coelicolor*, the functional reconstitution of CYP-sb21 for *in vitro* CsA hydroxylation is currently unclear (data not shown), probably due to an inappropriate supply of electron partners and/or a requirement of some unknown factors, and should be demonstrated unambiguously in the future. To further increase our understanding of the regio- and stereo-specific activities of CYP-sb21, the *in vitro* enzyme kinetics, three-dimensional structure, and protein engineering of CYP-sb21 should be studied. In conclusion, this is the first report that *S. benihana* CYP-sb21, belonging to the bacterial CYP107 family, is the key cytochrome P450 hydroxylase involved in regio-specific CsA hydroxylation at the 4th *N*-methyl leucine position.

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