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Heterologous Expression of Novel Cytochrome P450 Hydroxylase Genes from *Sebekia benihana*

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Abstract Actinomycetes are ubiquitous Gram-positive soil bacteria and a group of the most important industrial microorganisms for the biosynthesis of many valuable secondary metabolites as well as the source of various bioconversion enzymes. Cytochrome P450 hydroxylase (CYP), a heme-binding protein, is known to be involved in the modification of various natural compounds, including polyketides, fatty acids, steroids, and some aromatic compounds. Previously, six different novel CYP genes were isolated from a rare actinomycetes called *Sebekia benihana*, and they were completely sequenced, revealing significant amino acid similarities to previously known CYP genes involved in *Streptomyces* secondary metabolism. In the present study, these six CYP genes were functionally expressed in *Streptomyces lividans*, using an ermE* promoter-containing *Streptomyces* expression vector. Among six CYP genes, two *S. benihana* CYP genes (CYP503 and CYP504) showed strong hydroxylation activities toward 7-ethoxycoumarin. Furthermore, the recombinant *S. lividans* containing both the *S. benihana* CYP506-ferredoxin genes as well as the *S. coelicolor* feredoxin reductase gene also demonstrated cyclosporin A hydroxylation activity, suggesting potential application of actinomycetes CYPs for the biocatalysts of natural product bioconversion.

Key words: Cytochrome P450 hydroxylase, *Sebekia benihana*, coumarin, cyclosporin, heterologous expression

Streptomycetes and their physiologically related actinomycetes are ubiquitous Gram-positive soil bacteria and a group of the most important industrial microorganisms for the biosynthesis of many valuable secondary metabolites as well as the source of various bioconversion enzymes [1, 3, 6, 14]. Among various industrially valuable actinomycetes-derived enzymes, cytochrome P450 hydroxylase (CYP), a heme-binding protein, is involved in the modification of various natural compounds, including polyketides, fatty acids, steroids, and some aromatic compounds [5, 8, 13, 15, 16]. In general, the actinomycetes CYP is known to transfer to the substrate one oxygen atom from CYP-bound oxygen molecule and one hydrogen atom from NAD(P)H, which is regenerated by the ferredoxin-ferredoxin reductase electron transfer system [4, 9, 10]. One rare actinomycetes strain, *Sebekia benihana*, has been reported to be an important microorganism for natural product bioconversion, because this strain possesses unique regiospecific hydroxylation capabilities with cyclosporin, monensin, and nigericin [2, 7, 12, 17]. Previously, six different novel CYP genes have been isolated from *Sebekia benihana*, and they were completely sequenced, revealing significant amino acid similarities to previously known CYP genes involved in *Streptomyces* secondary metabolism [11]. In this short communication, we report the functional expression of *S. benihana* CYP genes in a heterologous host *S. lividans*, followed by bioconversion assay with putative substrates including 7-ethoxycoumarin and cyclosporin A, suggesting potential application of actinomycetes CYPs for natural production bioconversion.

S. benihana (KCTC 9610) obtained from 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, followed by cell harvest and total DNA isolation [3]. Based on the previously identified sequence information [11], each of

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Table 1. The sequences of PCR primer pairs for the amplification of *S. benihana* CYP genes.

CYP genes	Forward primer	Reverse primer
CYP501	5'-GGATCCTCTACATCTGTAGAGGATGC-3'	5'-AAGCTTTGCGAGCAGAGCTACCACAT-3'
CYP502	5'-GGATCCGAGTCAGATATCAAATTTCC-3'	5'-AAGCTTTCATCGGCCTTCCTGGGCGC-3'
CYP503	5'-GGATCCGATCAACACGGTGACAA-3'	5'-AAGCTTAACTGGAGGCAATGACC-3'
CYP504	5'-GGATCCTCTGGGAGGGAGTCGATCCC-3'	5'-AAGCTTTTCCGCGAACACAGGTTCGA-3'
CYP505	5'-TCTAGACACGCCGGAGAGGGTGA-3'	5'-AAGCTTAGGCTCGATCCGAGGCT-3'
CYP506	5'-GGATCCGGAGCGGAACGCGTAATATC-3'	5'-AAGCTTTCACCGGAGAGGAAGACCTG-3'

six *S. benihana* CYP genes was individually amplified by polymerase chain reaction (PCR) using the restriction-site-containing primer pairs (Table 1). All six PCR-amplified CYP genes included the putative upstream ribosome binding site, start codon, and stop codon sequences. Because four CYP genes (CYP502, 503, 504, and 506) were immediately followed by the unique ferredoxin genes at the 3' downstream region, these four PCR-amplified products also included ferredoxin genes at 3' downstream of CYP genes (Fig. 1A). Each of six PCR-amplified CYP genes was then cloned into the pGEM-T easy vector (Progema, U.S.A.), followed by complete sequence verification, and then finally subcloned into the *Streptomyces* expression vector pSE34, resulting in pESK50X-E1 (Fig. 1B). Each of six pESK50X-E1 plasmids was individually introduced into *S. lividans* TK21 *via* the polyethylene glycol (PEG)-mediated protoplast transformation method, followed by the tiostrepton (*tsr*) selection method [3].

The recombinant *S. lividans* strain containing pESK50X-E1 plasmid was cultured in 5 ml of GSMY at 28°C with constant shaking at 200 rpm for 3 days and then further cultured for 5 days after adding 7-ethoxycoumarin as hydroxylation substrate into the culture. It has been reported that 7-ethoxycoumarin is an excellent broad-specificity CYP substrate owing to its efficient oxidation to highly fluorescent 7-hydroxycouramin by many microbial CYPs [5]. To test whether *S. benihana* CYP genes are functionally expressed in *S. lividans* and efficiently oxidize 7-ethoxycoumarin to 7-hydroxycoumarin, 1 ml of each culture broth was extracted with 0.5 ml of ethyl acetate solvent, and the extract was then analyzed on thin-layer chromatography (TLC), using hexane-ethyl acetate (3:2) mixture as a running buffer. As shown in Fig. 2A, two recombinant *S. lividans* strains, containing pESK503-E1 and pESK504-E1 each, exhibited significant bioconversion activities toward 7-ethoxycoumarin, much higher than the residual background activities detected in all samples. These results imply that the *S. benihana* CYP genes are functionally expressed in a heterologous host, and the substrate specificities among *S. benihana* CYPs are rather diverse. As previously reported, the most interesting bioconversion capability present in *S. benihana* is the regiospecific hydroxylation of cyclosporin A [12]. To test whether the *S. benihana* CYP genes expressed in *S. lividans*

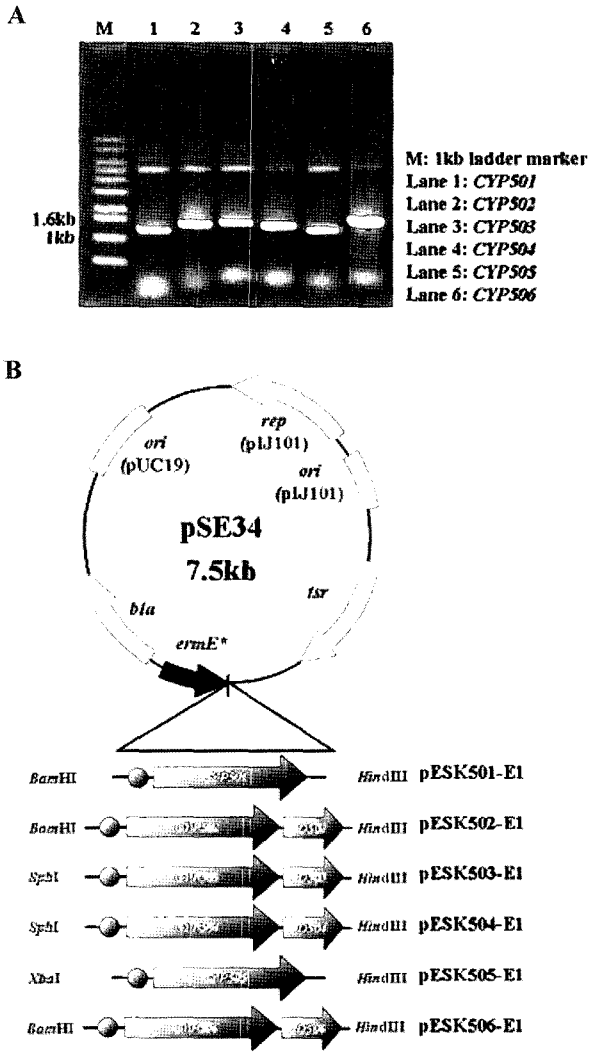

Fig. 1. A. Agarose gel electrophoresis of six PCR-amplified *S. benihana* CYP genes. B. The schematic map of expression vector pSE34 derivatives containing *S. benihana* CYP genes.

efficiently hydroxylate cyclosporin A, all six pESK50X-E1 containing recombinant *S. lividans* were cultured in the presence of cyclosporin A, and its bioconversion was analyzed by the HPLC assay method described elsewhere [12]. Unfortunately, all the six recombinant *S. lividans* strains containing pESK50X-E1 failed to exhibit detectable

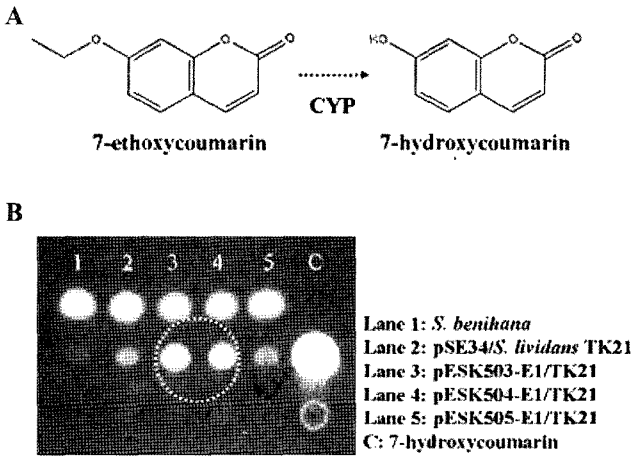

Fig. 2. A. Structures of 7-ethoxycoumarin and 7-hydroxycoumarin. B. TLC spotted with 0.5 ml of ethyl acetate extract with 1 ml of each recombinant *S. lividans* culture followed by hexane-ethyl acetate (3:2) elution and UV visualization. The dotted circle indicates the oxidized product, 7-hydroxycoumarin.

bioconversion capability with cyclosporin A (data not shown). Interestingly, however, clear bioconversion of cyclosporin A was observed when the *S. benihana* CYP506 gene was coexpressed with the *S. coelicolor* ferredoxin reductase gene in *S. lividans* (Fig. 3), indicating that the cyclosporin A hydroxylation requires not only a specific CYP-ferredoxin gene expression, but also a specific ferredoxin reductase gene expression for the completion of ferredoxin regeneration. In conclusion, the functional expression of *S. benihana* CYP genes in a heterologous host and successful bioconversion of putative substrates implies potential application of actinomycetes CYPs as a valuable source of biocatalysts involved in natural product bioconversion.

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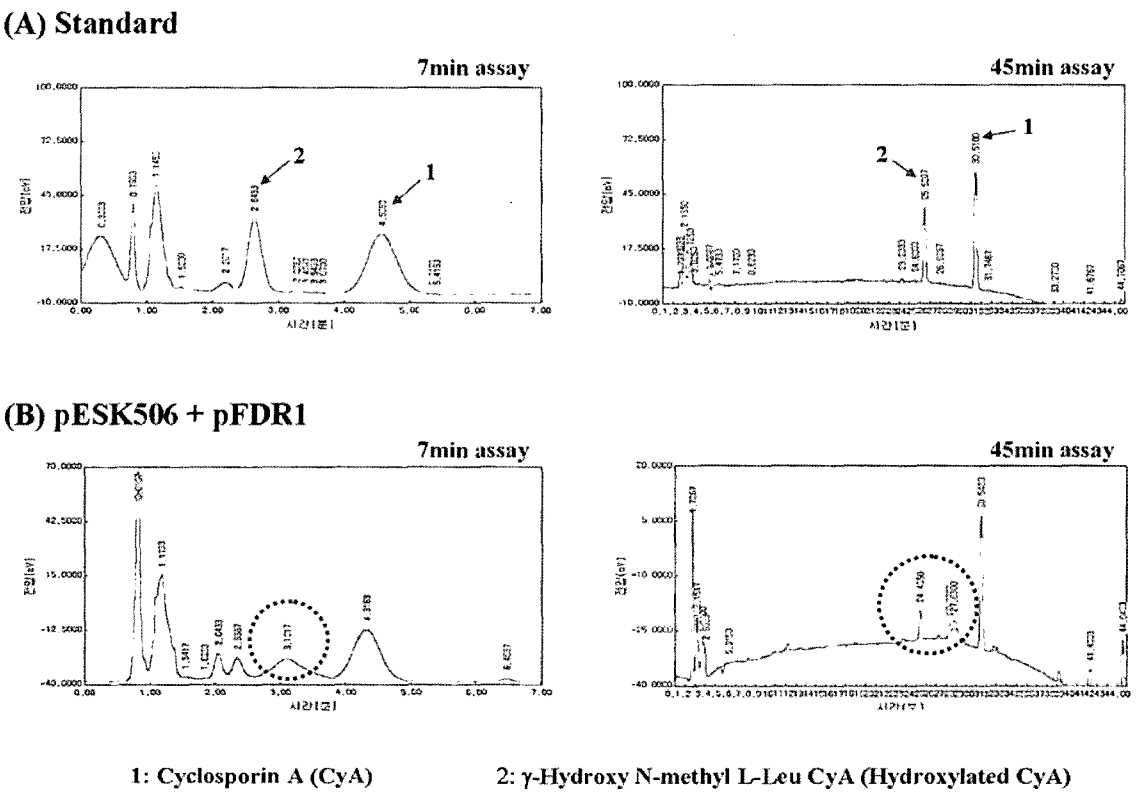

Fig. 3. HPLC chromatograms of cyclosporine A conversion with 7-min-assay (on left) and 25-min-assay (on right) whose detailed procedures are described elsewhere [12]. A. Standard cyclosporin. B. The extract from the *S. lividans* containing both pESK506 and pFDR1. The pESK506 is the pOJ446-derived cosmid containing *S. benihana* CYP506 gene [11], and pFDR1 is the pSE34-derived expression plasmid containing *S. coelicolor* feredoxin reductase gene [5]. The dotted circle indicates the hydroxylated form of cyclosporin A.

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