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Molecular Cloning and Analysis of the Gene for P-450 Hydroxylase from *Pseudonocardia autotrophica* IFO 12743

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A 4.8-kb DNA fragment encoding the P-450 type hydroxylase and ferredoxin genes was cloned from *Pseudonocardia autotrophica* IFO 12743 that can convert vitamin D_3 into its hydroxylated active forms. In order to isolate the P-450 gene cluster in this organism, we designed PCR primers on the basis of the regions of an oxygen binding site and a heme ligand pocket that are general characteristics of the P-450 hydroxylase. Sequencing analysis of the *Bam*HI fragment revealed the presence of four complete and one incomplete ORFs, named PauA, PauB, PauC, and PauD, respectively. As a result of computer-based analyses, PauA and PauB have homology with enoyl-CoA hydratase from several organisms and the positive regulators belonging to the *tetR* family, respectively. PauC and PauD show similarity with SuaB/C proteins and ferredoxins, respectively, which are composed of P-450 monooxygenase systems for metabolizing two sulfonylurea herbicides in *Streptomyces griseolus* PauC shows the highest similarity with another CytP-450$_{Sca2}$ protein that is responsible for production of a specific HMG-CoA reductase inhibitor, pravastatin, in *S. carbophilus*. Cultures of *Streptomyces lividans* transformant, containing the P-450 gene cluster on the pWHM3 plasmid, was unable to convert vitamin D_3 to its hydroxylated forms.

Key words: P-450 hydroxylase, ferredoxin, *Pseudonocardia autotrophica*, biotransformation

The P-450 type hydroxylase is very common to eukaryotic and prokaryotic systems and responsible for various metabolizing activities as a monooxygenase. The usefulness of the P-450 hydroxylase lies in their importance their bioconverting activities of water insoluble chemicals into soluble ones by the introduction of mono-oxygen via hydoxylation reaction (Raucy and Allen, 2001). Recently, many of the P-450 hydroxylases have been reported in prokaryotes including Actinomycetes. Actinomycetes have been intensively studied for their importance as industrial microorganisms in producing various kinds of antibiotics, bioactive subtances and enzymes. By an appropriate treatment of the P-450 hydoxylase gene, many noble compounds are produced in Actinomycetes. For example, a noble amphotericin derivative from *Streptomyces nodosus*, a piramicin derivative from *Streptomyces natalensis*, a 16-O-desmethyl-27-desmethoxyrapamycin from *Streptomyces hygroscopicus* ATCC29253 were reported recently (Caffrey *et al.*, 2001; Mendus *et al.*, 2001), and engineering of the heterologous cytochrome P450 is a promising field of research for the application in noble metabolites and pollutant degradation.

Pseudonocardia autotrophica IFO12743 is a group of actinomycetes that has bioconversion activity of vitamin D_3 into active forms (Kawauchi *et al.*, 1994). In mammals and birds, vitamin D_3 is converted to 25-hydroxyvitamin D_3 [25(OH)D_3] in the liver. Then 25(OH)D_3 is converted to 1α, 25-dihydroxyvitamin D_3 [1α, 25(OH)$_2D_3$] in the kidney (Itoh *et al.*, 1995; Sawada *et al.*, 2000). These hydroxylating reactions are catalyzed by P-450 hydroxylase. Because of the industrial usefulness of these P-450 hydroxylases that can convert vitamin D3 into its active forms, cloning of the P-450 hydroxylase gene was performed from *P. autotrophica* IFO12743. In this study, we have cloned the P-450 gene cluster from *P. autotrophica* IFO12743 and report its genetic information.

Materials and Methods

Bacterial strains and plasmids

P. autotrophica IFO12743 was obtained from the Institute

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for Fermentation, Osaka, Japan. *S. lividans* TK24 was obtained from the University of Tokyo, Japan. *E. coli* strain DH5α was used for subcloning. The *Streptomyces-E. coli* shuttle vector pWHM3 was obtained from C. R. Hutchinson (University of Wisconsin, Madison, WI, USA).

Media and culture conditions

E. coli DH5α was maintained on M9 minimal agar and cultured in LB medium at 37°C with agitation (Sambrook *et al.*, 1989). *Streptomyces* and *Pseudonocardia* strains were maintained on R2YE agar and *S. lividans* TK24 grown in R2YE liquid broth at 28°C was used for the preparation of protoplasts and the isolation of plasmid DNAs (Chi *et al.*, 2001).

Enzymes and chemicals

Restriction endonucleases and other DNA modifying enzymes were purchased from Takara Shuzo Inc, Japan and other fine chemicals were from Sigma Chemical Co. The primers for PCR was obtained from Atman Bio-Science Inc., Korea.

DNA manipulations

DNA preparation and manipulation was performed by the method of Maniatis *et al.* in *E. coli* (Sambrook *et al.*, 1989) and *Streptomyces* (Hopwood *et al.*, 1985). DNA samples were digested with restriction endonucleases and ligated with T4 DNA ligase according to the supplier's recommendations. Plasmid DNAs and their digests were analyzed by horizontal agarose gel electrophoresis with TAE buffer system (Sambrook *et al.*, 1989).

Transformation procedure

Competent cells of *E. coli* strains for transformation were routinely prepared according to the frozen storage protocol and transformation was done by the method described by Maniatis (Sambrook *et al.*, 1989).

Protoplasts of *S. lividans* TK24 were prepared as described by Hong *et al* (Hong *et al.*, 1991). Cells were grown in 100 ml of R2YE containing 0.5% glycine in a 500 ml baffled flask and incubated for 18 to 24 h. The obtained cells were treated with 20 ml of P buffer containing 80 mg of lysozyme, and the resulting protoplasts were suspended in P buffer at a concentration of ~10^9/ml and frozen at -70°C. *Streptomyces* protoplasts were transformed as described by Hong *et al.* (1991) by using 100 μl of protoplasts (~10^8 cells), 1 μg of plasmid DNA in 20 μl of TE buffer, and 500 μl of 25% PEG1000 in P buffer. Samples (100 μl) were plated in 2.5 ml of 0.6% soft R2YE agar on R2YE regeneration plates. After incubation at 28°C for 18 to 24 h, the plates were overlaid with 2.5 ml of 0.6% soft R2YE agar containing 25 μg/ml of thiostrepton. Transformants were visible after incubation for an additional 3 to 5 days at 28°C.

Amplification of the P-450 hydroxylase gene fragment by PCR

P. autotrophica IFO 12743 was the original source of genomic DNA for the cloning experiments. The comparison of the sequences of the known P-450 hydroxylases from *S. carbophilus, S. griseus, S. griseolus*, and *P. autotrophica* revealed high similarity in the region for an oxygen binding site and a heme ligand pocket. On the basis of the consensus sequences of P-450 hydoxylase from Amy (D26543), SuaC (M32238), SubC (M32239), Sca2 (D30815), and Soy (X53501), and the codon bias of *Streptomyces* genes, the primers were designated as 5'-TXCTXCTXATCGCXGGXCACGAGAC-3', which corresponds to the conserved region of the oxygen binding site, and 5'-GCXAGGTTCTGXCCXAGGCACTGGTG-3', which corresponds to the complementary sequence of a conserved region in the heme ligand pocket site (X=G+C). These primers were used to amplify DNA fragments from the chromosomal DNA of *P. autotrophica* IFO 12743.

The PCR mixture contained 10X PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM $MgCl_2$) supplied by Takara Co. Ltd. (Japan). Taq polymerase (2U, Takara) and approximately 100 ng target DNA were added in a final reaction volume of 50 μl. Amplification was performed in a thermal cycler (model 480, Perkin Elmer Cetus, CT, USA) by denaturing the samples at 94°C for 4 min, subjecting them to 30 cycles of denaturing (98°C, 20sec), annealing (67°C, 1 min), and then by elongating at 72°C for 10 min. The 350 bp PCR products were recovered using 1.5% agarose gel electrophoresis and ligated into pT7Blue (Novagen, Madison, WI, USA).

Analysis of the PCR products

PCR fragments obtained from various reactions were sequenced. The nucleotide sequences of both strands were determined using an ABI model 373 DNA sequencer (Applied Biosystems Inc., USA). The deduced amino acid sequences of the isolated fragments revealed remarkable similarity to the P-450 hydroxylases isolated from *S. carbophilus, S. griseus, S. griseolus*, and *P. autotrophica.*

Nucleotide sequence determination of the cloned P-450 hydroxylase gene

One clone with a 4.8-kb insert was selected as a candidate for P-450 hydroxylase gene. The DNA fragments were subcloned into pBluescript and sequenced by Automatic sequencer with the synthesized primers. In part, DNA sequencing was performed manually on single-stranded templates prepared in M13mp18/19 by the dideoxy method, using [α-^{35}S] dATP (1000 μCi/mmol; Amersham) and sequenase 2.0 (U.S. Biochemicals) according

to the instructions of the manufacturer. To reduce compression, sequencing reactions were carried out with a 7-deaza-dGTP nucleotide mixture. The nucleotide and deduced amino acid sequence homology with other P-450 hydroxylase genes was analyzed with Blast algorithm using the Entrez database (National Center for Biotechnology Information, USA). A multiple amino acid sequence alignment was generated using the Clustal X program (Higgins and Sharp, 1988).

Results and discussion

Isolation of the P450 gene cluster from P. autotrophica IFO 12743

The PCR products amplified using the degenerate primers were used as the probe in the Southern hybridization. The fragment produced by the PCR (0.5 kb) with the two primers were labelled with biotin using the Bionick Labelling System (Life Technology, Rockville) and then used as the probe for the Southern hybridization. *P. autotrophica* IFO 12743 genomic DNA (10 μg) completely digested with *Bam*HI was used for the Southern hybridization. After the identification of the position of the hybridization signals, the corresponding region of digested *P. autotrophica* IFO 12743 genomic DNA was isolated by an agarose gel elution using Geneclean III Kit (BIO101 Inc., Carlsbad), ligated with pBluescript II SK (+) vector digested with *Bam*HI, and then transformed in *E. coli* DH5α. About 10^3 of independent clones were isolated using alpha complementation. A subgenomic library (about 10^3 independent clones), consisting of 4.0 to 6.0-kb *Bam*HI DNA fragments of *P. autotrophica* genomic DNA, was screened by the PCR mediated method (Hyun *et al.*, 2000). As a result of PCR screening, we isolated one positive clone, pJM16, which was shown to contain a 4.8-kb DNA fragment. A restriction map of the cloned region is shown in Fig. 1.

Nucleotide sequence analysis of the pauA-pauD region

The 4.8-kb *Bam*HI fragment was cloned and sequenced (Fig. 2). The entire fragment was found to be 4,770 bp long. Frame-plot analysis showed the presence of four open reading frames (ORFs), named PauA, PauC, and PauD, localized on one DNA strand and one open reading frame, named PauB, on the opposite strand. The salient features of this region, deduced from the nucleotide sequence, are described below and summarized in Table 1. The translational start points in the other genes were unambiguous, and the ORFs in *pauA, pauB, pauC,* and *pauD* are preceded by good ribosome binding sites (RBS). The *pauA* start codon overlaps with the incomplete orf stop codon (ATGA). The *pauC* and *pauD* genes are separated by 6 bases (TGAGGGCCCATG).

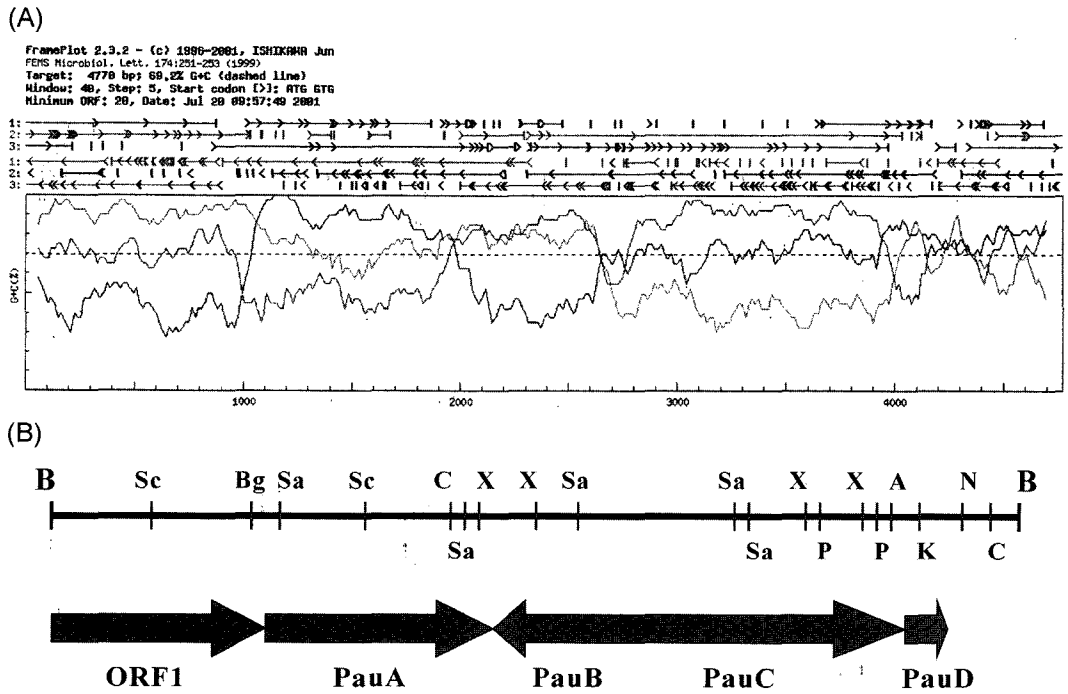

Fig. 1. Frame anaysis, restriction map and genetic organization of the P-450 locus. (A) FRAME analysis of the 4.8-kb DNA fragment including P-450 gene cluster. The arrowheads on the lines above and below the graph indicate translational start codons and their orientations; vertical bars crossing the lines represent translational stop codons. (B) Restriction map of P-450 locus. The thick solid bar shows the extent of the sequenced DNA fragment. The sizes and orientations of the genes indicated by arrows were deduced from the nucleotide sequence. Restriction site abbreviations are: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; K, *Kpn*I; N, *Nco*I; P, *Pst*I; Sa, *Sal*I; Sc, *Sca*I; X, *Xho*I.

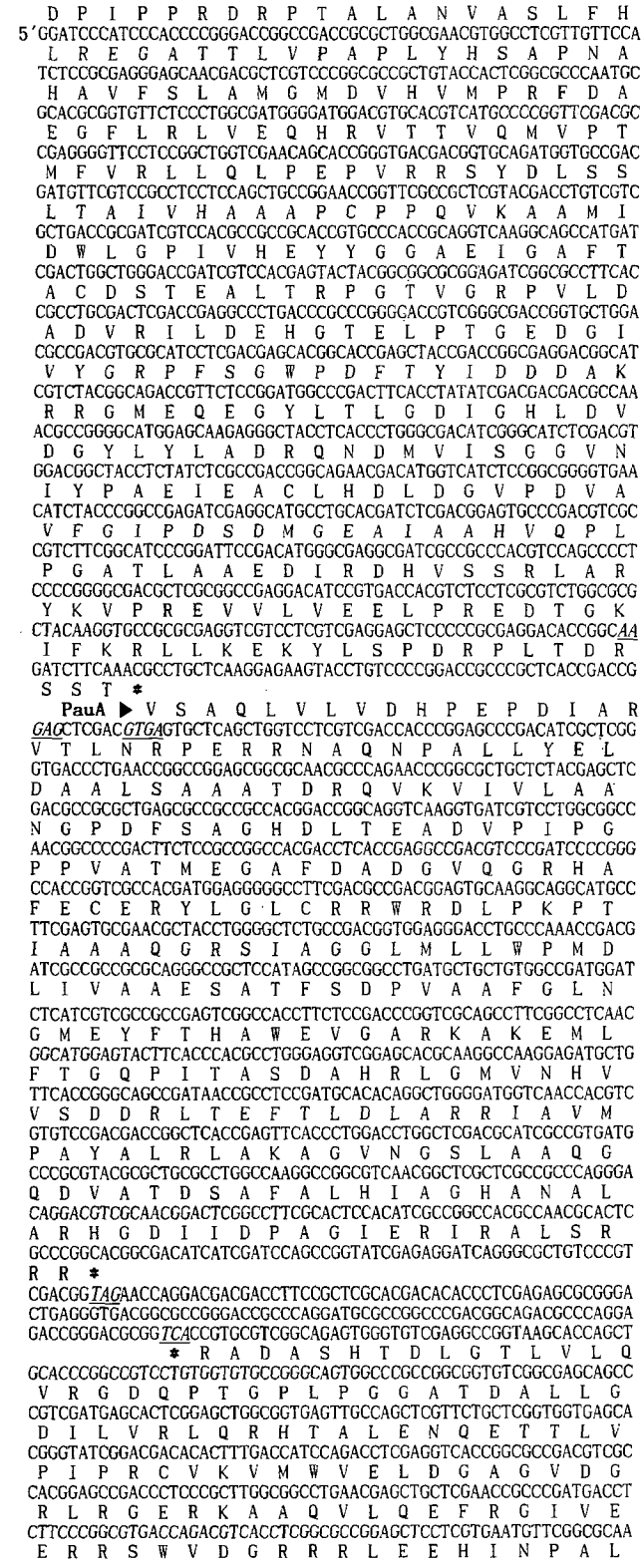

Fig. 2. Nucleotide sequence of P-450, ferredoxin and flanking open reading frames, and amino acid sequence of predicted protein products. The DNA sequence is shown, along with the single-letter amino acid codes above the open reading frames and all reading rightward, excluding the PauB. The probable start codon, termination codon, and ribosomal binding sites are also underlined and italicized.

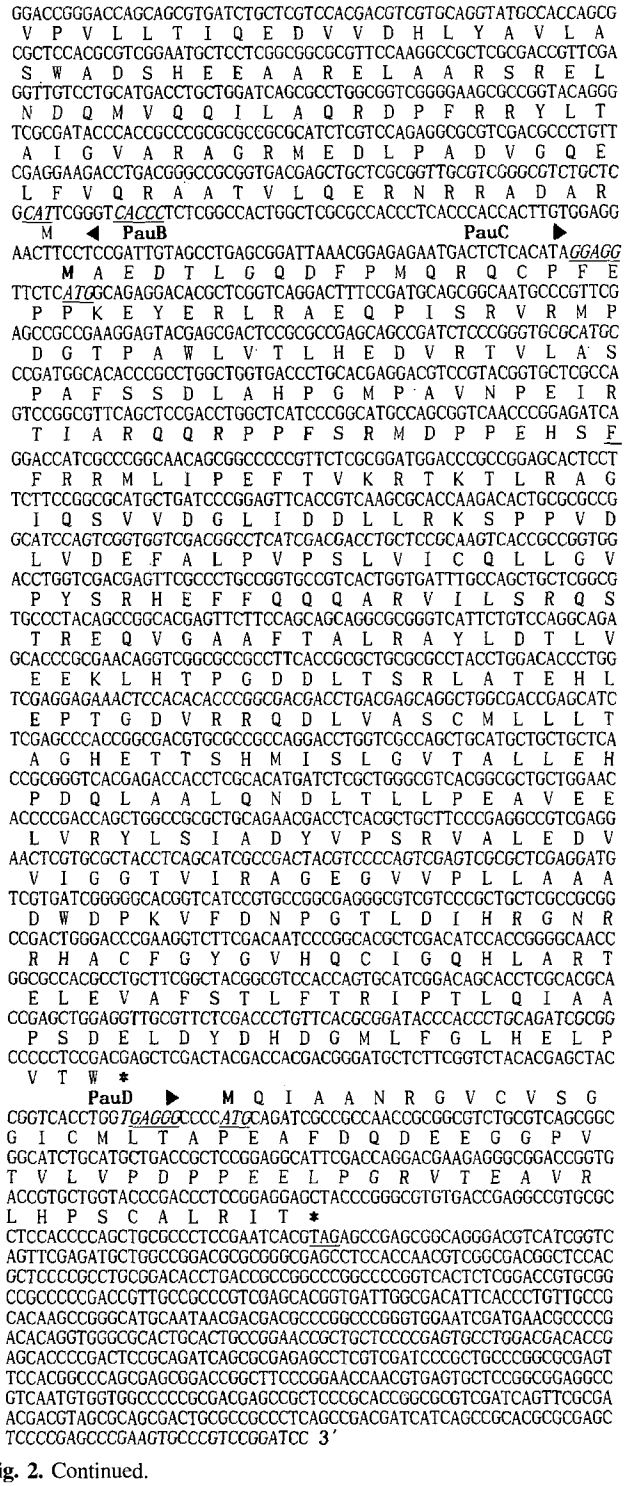

Fig. 2. Continued.

The first open reading frame (*pauA*) shows a GTG start codon at nucleotide (nt) 1030 and ends at nt 1869. The resulting polypeptide of 279 amino acids (aa), PauA (calculated molecular mass, 29.9 kDa), belongs to the enoyl-CoA hydratase (ECH) family, including an ECHH (for enoyl-CoA hydratase homolog) from *Mycobacterium tuberculosis* H37rV (43.1% identity), possibly involved in

Table 1. Relevant features of the P-450 gene cluster region deduced from its DNA sequence

ORF	RBS	Start/Stop	G+C	Residues/MW	Predicted functions
ORF1	–	/TGA[1033]	68.8	343/37.6	Substrate-CoA ligase
PauA	AAGAA	[1030]GTG/TAG[1869]	70.7	279/29.9	Enoyl-CoA hydratase
PauB	GAGGG	[2644]ATG/TGA[1994]	69.2	216/24.2	TetR type regulator
PauC	GGAGG	[2766]ATG/TGA[3974]	68.2	402/44.9	P-450 hydroxylase
PauD	GAGGG	[3982]ATG/TAG[4173]	69.8	63/6.6	Ferredoxin

Numbers indicate the first and last nucleotide of the start and stop codons, respectively.

fatty acid oxidation (Cole *et al.*, 1998), and the *orf3* product (43.2% identity) from *Comamonas testosteroni* involved in testosterone degradation (Horinouchi *et al.*, 2001).

Upstream of PauA, we identified the 3'-end of a possible open reading frame (nt 1-1033). Comparison of this incomplete orf with protein databases showed similarity with long-chain fatty-acid CoA ligases (EC 6.2.1.3) and 4-coumarate CoA ligases (EC 6.2.1.12). Actually, the best score (43.1% identity) was with *M. tuberculosis* fadD4 protein (Cole *et al.*, 1998), which in turn shares 24.6% identity with 4-coumarate CoA ligase (P41636) (Zhang *et al.*, 1997). All these enzymes contain the putative AMP-binding domain signature.

The second open reading frame, *pauB*, shows a GTG start codon at nt 2644 and ends at nt 1994. The resulting polypeptide of 216 aa, PauB (calculated molecular mass, 24.2 kDa) revealed a high homology with the positive regulators belonging to the TetR family (Kojic and Venturi, 2001). The best score (38.5% identity) was with the putative regulatory protein from the soil bacterium *S. coelicolor* A3(2) (Bentley *et al.*, 2002). The amino acid sequences of PauA and PauB were aligned with other paralogue enzymes reported in other bacterial strains as shown in Fig. 3 and Fig. 4.

The third open reading frame (*pauC*) shows a GTG start codon at nt 2766 and ends at nt 3974. PauC encodes a protein of 402 amino acids with a predicted mass of 44.9 kDa and a calculated pI of 5.15. A BLASTP (Altschul *et al.*, 1990) search showed that this gene product, P-450$_{pau}$, has high sequence similarity (36 to 48% identity and 50 to 61% similarity) to cytochrome P-450 monooxygenases from several microorganisms. The greatest similarities are to the cytochrome P-450 proteins encoded by the sca2 gene (48% identity, 61% similarity) from *S. carbophilus* (Watanabe *et al.*, 1995; Watanabe and Serizawa, 1998) and by the *subC* gene from *S. griseolus* (47 and 63%). Some of the homologous P-450s are involved in the biosynthesis of secondary metabolites in streptomycetes,

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PauA     --------------------SAQLVLVDHPEPD---IARVTLNRPERRNAQNPALLYELD
EchA13   MFVGRVGPVDRRSDGERSRRPREFEYIRYETIDDGRIAAITLDRPKQRNAQTRGMLVELG
Cte3     -----MGDASSEFVTREDKAVYETDEPVLYSVDNG-IATVTMNRPTFNNVQNSQMTYALD
                            :         *   ** :*::**  .*.*.  :   *.

PauA     AALSAAATDRQVKVIVLAANGPDFSAGHDLTEADVPIPGPPVATMEGAFDAD-----GVQ
EchA13   AAFELAEADDTVRVVILRAAGPAFSAGHDLGSADDIRERSPGPDQHPSYRCNGATFGGVE
Cte3     AAFRKATDDDSVKVIVLRGEGKHFSAGHDIGTPGRDINKSFDRAHLWWDHTNKP---GGE
         **:  *  *  *:*::* . *  ******:  ..     .            :     * :

PauA     GRHAFECERYLGLCRRWRDLPKPTIAAAQGRSIAGGLMLLWPMDLIVAAESATFSDPVAA
EchA13   SRNRQEWHYYFENTKRWRNLRKITIAQVHGAVLSAGLMLAWCCDLIVASEDTVFADVVGT
Cte3     QLFAREQEVYLGMCRRWREIPKPMIAMVQGACVAGGLMLAWVCDLIVASDDAFFQDPVVR
              * . *:   :***:: *  ** .:*  ::.**** *  *****::.: * * *

PauA     -FGLNGMEYFTHAWEVGARKAKEMLFTGQPITASDAHRLGMVNHVVSDDRLTEFTLDLAR
EchA13   RLGMCGVEYFGHPWEFGPRKTKELLLTGDCIGADEAHALGMVSKVFPADELATSTIEFAR
Cte3     -MGIPGVEYFAHAHELHPRIAKEFLLLGERMPAERAYQMGMVNRVVPRAELQDQVYAMAQ
          :*: *:*** *. *. .* :**:*: *: : *. *: :***.:*..  .*   .  :*:

PauA     RIAVMPAYALRLAKAGVNGSLAAQGQDVATDSAFALHIAGHANALARHG----------D
EchA13   RIAKVPTMAALLIKESVNQTVDAMGFSAALDGCFKIHQLNHAHWGEVTGGKLSYGTVEYG
Cte3     RMAAQPRLGLALTKMVVNKAEELQGLRSTMEMAFGYHHFAHAHSQAMGMG-------QLG
         *:*  *  .  * *  ** :     *   : :.*  *   **:               .

PauA     IIDPAGIERIRALSRRR-
EchA13   LEDWRAAPQIRPAIKQRP
Cte3     GQDARSMAKANKEESKA-
           *  .  : .    :
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Fig. 3. Alignment of the deduced amino acid sequence of *P. autotrophica* PauA protein with two other enoyl-CoA hydratase (ECH) families. The enoyl-CoA hydratase/isomerase family (ECH) signature is located in 16 to 198 aa. This family contains a diverse set of enzymes including enoyl-CoA hydratase, napthoate synthase, carnitate racemase, 3-hydoxybutyryl-CoA dehydratase, dodecanoyl-CoA delta-isomerase, EchA13, *Mycobacterium tuberculosis* H37rV; Cts3, *Comamonas testosteroni*. Where the consensus sequence is highly conserved, the amino acids are asterisked.

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PauB       ----------------------------------------MRADARRNREQLVTAARQVF
AL355832   FPPRIVNPVVTPVTDTLTDTTDTKEYAVQTAIPDQRKVARPRADALRNRERIVLAAREMF
AL450165   -----------------------------MRVVTGHLPHPLRSDARDNRERILDAARALF
                                                   *:**  ***::: ***  :*

PauB       LEQGVDAPLDEMRGARAVGIATLYRRFPDRQALIQQVMQDNLERSRAALERAAEEHSDAW
AL355832   VEHGPDVPLDDVARRAGVGNATVYRNFPDRDALVREVVCSVMDRTARAAELALAETGDAF
AL450165   ATEGLRVPMREVARRAGVGPATLYRRFPTKRELVTEAFTDQLRACRTIVDEGLAHP-DPW
             .*  .*: ::    .** **:**.** :  *: :.. . :       : .  . *.:
             Helix-turn-helix motif region

PauB       SALVAYLHDVVDEQITLLVPVLAPNIHEELRRRGDVWSRREEVIGRFEQLVQAAKREGRL
AL355832   EALERFVHASADERISALCPMVASTFDQHHP---DLEAARGRVERLVAEVMDRAKAAGQL
AL450165   AGFCLVVEKTCELHARHLG--FTEAFMAAYPEAMDFAAARAYSLKSAGELARRAREAGGL
            .:   :.   : :   *  .: :          *. : *       ::   *:  * *

PauB       RGDVGAGDLEVWMVKVCRPIPVLTTEQNELATHRQLRVLIDGLLADTAGGPLPGTPQDGR
AL355832   RGDVGVGDLMIAAAQLSRPPAGTGCVNADRFVHRHLQLFLDGLRAP-ARSVLPG------
AL450165   RPDFVLDDLILMIMAN-RGIHALSTAARVAASRRFAALVIQAFRASPEHTRLPP------
           * *.  .** :        *                 :*   :.::.: *      **

PauB       VQLVLTGLDTHSADAR
AL355832   AAVTLEDLRRPCDQ--
AL450165   VPRLAPG---------
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Fig. 4. Amino acid comparison of three *tetR* type regulator sequences. The top sequence is from this work. Others are the *tetR* regulator gene product from *S. coelicolor* A3(2). Identical amino acids are marked by asterisks. Dashes indicate gaps introduced for the optimization of the alignment. The predicted helix-turn-helix region involved in DNA binding is also underlined.

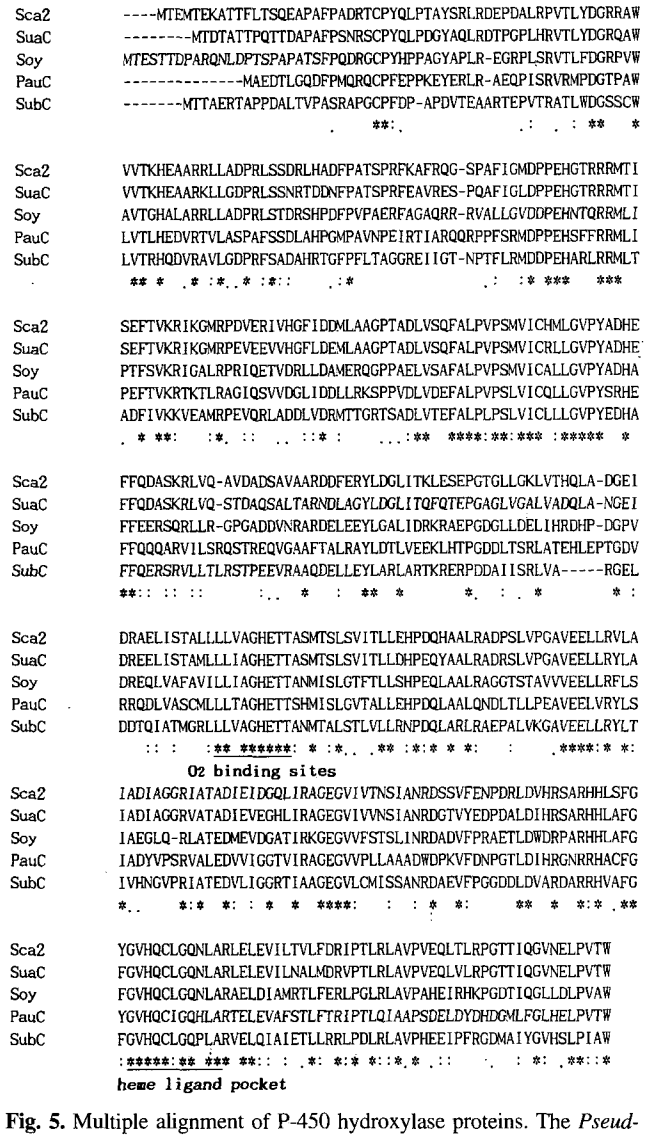

Fig. 5. Multiple alignment of P-450 hydroxylase proteins. The *Pseudonocardia* PauC sequence was aligned with the sequences of several P-450 hydroxylase enzymes in the databases by using the Clustal alignment program (Higgins and Sharp, 1988). Hyphens indicate gaps introduced to give the best sequence alignments. Identical amino acids are marked by asterisks. Homologous regions in the amino acid sequence of the O_2-binding site and heme-legand pocket of P-450 hydroxylases are underlined, and the underlined regions were used to design oligonucleotide primers for the amplification of P450 hydroxyase gene. The sources of amino acid sequences are as follow: *S. carbophilus* (Sca2); *S. griseus* (Soy); *S. griseolus* (SuaC and SubC).

including *S. tendae* (nikkomycin) (Bruntner *et al.*, 1999), *S. noursei* (nystatin) (Brautaset *et al.*, 2000), *S. natalensis* (pimaricin) (Aparicio *et al.*, 2000), and *S. avermitilis* (avermectin) (Omura *et al.*, 2001). Two highly conserved regions of cytochrome P-450 proteins exist in $P\text{-}450_{pau}$ (Fig. 5). The O_2 binding motif is present as LLLTAGHET, including the invariant L-237, L-238, A-240, G-241, H-242, and T-244. A very strongly conserved heme-binding pocket, HQCIGQHLAR, is evident and includes the

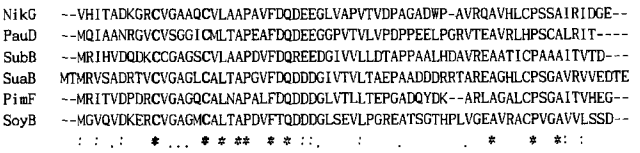

Fig. 6. Alignment of the amino acid sequences of PauD and other ferredoxins. The amino acid sequences of NikG (Bruntner *et al.*, 1999), SuaB (Omer *et al.*, 1990), SubB (Omer *et al.*, 1990), PimF (Mendes *et al.*, 2001), and SoyB (Trower *et al.*, 1993) were initially matched with PauD protein using the multiple sequence alignment CLUSTAL program, after which the resulting comparisons were refined manually (Higgins and Sharp, 1988). The multiple alignment between Fdpau and homologous ferredoxins revealed the high degree of conservation of 2 cysteine residues essential for coordination to iron at positions 10 and 16.

invariant H-349, Q-350, L-352, G-353, Q-354, L-356, A-357 residues as well as C-351 that coordinates to the heme iron. *pauD* (*fd*) consists of 189 nucleotides and starts from a GTG that is located 6 bp downstream of pauC (cyp) and is preceded by a presumed RBS (GAGGG). *pauD* (*fd*) encodes a protein, Fd_{pau}, of 63 amino acids with a molecular mass of 6.6 kDa and a calculated pI of 4.27. A database search showed that the deduced amino acid sequence of Fd_{pau} has a significantly high homology to the group of [3Fe-4S] ferredoxins from several *Streptomyces* spp. The greatest similarity (64%) was found to ferredoxin-1 from *S. griseolus* containing a [3Fe-4S] cluster (Omer *et al.*, 1990). The multiple alignment between Fd_{pau} and homologous ferredoxins revealed the high degree of conservation of 2 cysteine residues essential for coordination to iron at positions 10 and 16 (Fig. 6). As observed in several other organisms, the *P. autotrophica* ferredoxin gene is located downstream of and adjacent to the P-450 gene. This arrangement is highly suggestive that this is the in vivo electron transport protein functionally associated with $P\text{-}450_{sca}$.

Bioconversion experiment by using heterologous expression of P450 gene cluster in S. lividans

Since *P. autotrophica* IFO12743 can convert vitamin D_3 to 1α-hydroxyvitamin D_3 and 25-hydroxyvitamin D_3, and P-450 type hydroxylase was involved in this activity (Kawauchi *et al.*, 1994), we undertook the bioconversion experiment as follows. Plasmid pJM16, which contained the P-450 gene cluster of *P. autotrophica,* was digested with *Bam*HI, and a 4.8-kb fragment was isolated and cloned into the *Bam*HI site of *E. coli-Streptomyces* shuttle plasmid pWHM3, generating plasmid pW16. Plasmid pW16 and the parent plasmid pWHM3 were then transformed into protoplasts of *S. lividans*, and transformants were selected with thiostrepton (Sigma) as described previously (Chi *et al.*, 2001).

Each transformant was cultured in the medium (pH 7.2) containing 1.5% glucose, 1.5% Bacto-soyton (Difco), 0.5% corn steap liquor, 0.5% NaCl, and 0.1 mg/ml of

vitamin D_3 for 72 h at 30°C. After fermentation, the culture broth was extracted with methanol/dichloromethane (2:1) and the dichloromethane layer was recovered and dried in a rotary evaporator. The resulting residue was dissolved in 12% 2-propanol in hexane and analyzed by high-performance liquid chromatography (HPLC). The analytical conditions were as follows: Zorbax-SIL column (4.6× 250 mm, Du Pont) at a flow rate of 1.5 ml per min with 12% 2-propanol in hexane. However transformants harboring pW3 as well as pWHM3, were unable to generate new peaks above the HPLC conditions, which indicated that the cloned fragment did not contain the genes for vitamin D_3 hydroxylases.

Concluding remarks

The genus *Streptomyces* is a rich source of cytochrome P-450 monoxygenase enzymes that are involved in a wide variety of xenobiotic transformation reactions. In *S. carbophilus* P-450_{sca} hydroxylates compactin to pravastatin, a tissue-selective inhibitor of 3-hydroxy-3-methylglutamyl-coenzyme A reductase (Watababe *et al.*, 1995; Watanabe and Serizawa, 1998). The sulphonyl urea herbicide is metabolized by two inducible *S. griseolus* P450s, P450SU1 (CYP105A1) and P450SU2 (CYP105B1) (Omer *et al.*, 1990). *S. griseus* contains a cytochrome P450 that is inducible by the isoavenoid genistein present in soyaour (Trower *et al.*, 1993). Extracts of this organism, prepared from soyaour-induced cells and supplemented with spinach ferridoxin and ferridoxin-NADPH reductase are capable of aromatic benzylic and alicyclic hydroxylation (Trower *et al.*, 1988).

Since P-450 hydroxylase typically do not have stringent substrate specificities, the genes encoding P-450 hydroxylases may take advantage of a biotransformation. Therefore, the determination of the P-450 primary sequence may be an important step towards our goal to bind and transform a various range of xenobiotic substates. Although P-450 gene cluster of *P. autotrophica* IFO 12743 had no capacity for the bioconversion of vitamin D_3, we expect that its substrates will be elucidated by several biotransformation programs, and moreover the P-450 system of this organism will be a valuable source to construct the new and/or chimeric enzymes by using molecular evolution and genetic engineering in the near future.

Nucleotide sequence accession number

The nucleotide sequence obtained in this study has been assigned GenBank accession no. AF525299.

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